

The Regulation of Yeast Sporulation.

Jane H. Kinnaird.



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Declaration.

I declare that this thesis has been composed by myself  
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### Summary.

Mutants altered in regulation of sporulation were examined with the aim of elucidating the mechanism of initiation of sporulation. These mutants are unusual in that they fail to grow, but sporulate extensively in the presence of a rich nitrogen source. This phenotype was observed with all non-fermentable carbon sources except ethanol. It appeared that the nitrogen repression control on sporulation might be defective. However, no effect of this mutation was found on enzymes which are subject to regulation by ammonium<sup>ion</sup>. This together with the observed variability of the mutant phenotype indicates that the primary alteration is not in nitrogen repression although this can under certain circumstances be overruled.

Ammonium<sup>ion</sup> produced a much greater inhibition of sporulation than did single amino acids. Experiments with a non-metaboliseable analogue, methylamine, pointed to ammonium as the effector of repression.

The spd1 phenotype could result from abnormal energy metabolism. Studies of carbon metabolism did not reveal any major differences, although it remains possible that the spd1 phenotype could arise from a defect not yet established.

Certain revertants of spd1 mutants regained the ability to grow on non-fermentable carbon sources but were deficient in sporulation. The reversion is in a single gene unlinked to the spd1 locus and recessive to the wild type. These strains acquired an abnormal pseudo-mycelial appearance in stationary phase and lost viability rapidly.

It is postulated that initiation of sporulation is not directly controlled by either carbon or nitrogen source but by some indicator of metabolic rate which interacts with a protein regulating the

transition between mitotic and meiotic division. Either metabolism is altered in the spd1 mutants or a defect in this receptor protein reduces its affinity for the effector molecule. spo0 revertants appear unable to arrest in G<sub>1</sub> and low viability is due to an abnormal and abortive cell cycle in stationary phase.

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## Chapter 1.

### INTRODUCTION

Spore formation in microorganisms results in a dormant structure differing morphologically and biochemically from the vegetative, dividing, state. The regulatory events which govern the transition from a programme of growth and division to one of development or differentiation have intrigued biologists for many years. Inherent in the study is the concept that the regulatory mechanisms involved may be fundamental to cell development in all other systems.

Sporulation in bacteria, mainly Bacillus, has been extensively studied over the years (for reviews see Piggot and Coote, 1976, Mandelstam, 1969) and there is a considerable volume of information available on the morphological and biochemical events which occur during sporulation. It is only fairly recently, however, with the development of better techniques for genetic study of mutants, that the goal of establishing the basic control mechanisms underlying spore formation has become a more viable proposition.

In contrast, however, yeast sporulation has not been subject to the same rigorous investigation in the past, although in recent years it has become a more popular system for study. In many ways a study of sporulation in yeast has advantages. Yeast are eukaryotes with most of the features of higher cells such as a nucleus, mitochondria, organised mitotic and meiotic division processes and DNA binding proteins. It is easy to construct diploids therefore genetic analysis is simplified. This contrasts with Bacillus where genetic relationships have to be studied by transformation and transduction techniques which are complex

and are limited in their application. More recently it has been possible to construct merodiploids which should prove useful although stability problems exist in this analysis.

Study of yeast sporulation, however, suffers from a lack of knowledge of early, specific sporulation events which can be measured easily such as antibiotic production or alkaline phosphatase production in Bacillus. Premiotic DNA synthesis and meiotic recombination are the earliest known specific markers and these occur some time after initiation.

### Morphology of sporulation in *Saccharomyces cerevisiae*.

Yeast sporulation, like bacterial sporulation, involves the formation of endospores within a "mother" cell. However, in *Bacillus* only one spore is formed inside each cell whereas in yeast, typical sporulation involves formation of four spores within the mother cell or ascus. These ascospores are the products of meiotic division of a diploid zygote.

### Life cycle.

The life cycle of *Saccharomyces cerevisiae* is shown in Figure 1.1. Zygote formation occurs when cells of opposite mating type fuse. This is followed by nuclear fusion. Diploid zygotes, like haploid cells, can reproduce mitotically by budding during vegetative growth but only diploid cells can undergo meiotic division which is a central feature of sporulation.

There is a great difference in the organisation of meiosis and mitosis as both are essentially very different processes although possessing many features in common. During meiosis for example chromosomes pair and recombination occurs at a high frequency. Although mitotic recombination can occur its frequency is very low compared to that found during meiosis (Mortimer and Hawthorne, 1969). These different processes must require the synthesis of specific proteins. Obviously the regulatory mechanisms governing the switch from mitotic to meiotic division must be inherent in the control of sporulation.

### Cytological changes during sporulation.

The work of Moens (1971) and Moens and Rapport (1971) established the sequence of cytological events occurring during meiosis and sporulation in *Saccharomyces cerevisiae* and

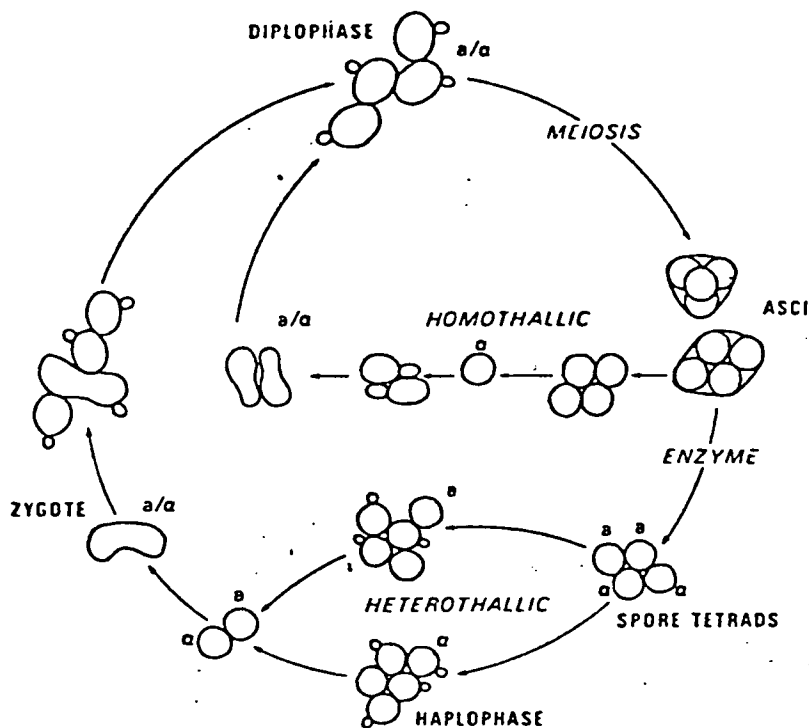


Figure 1.1

Life cycle of *Saccharomyces* species. The diagram illustrates the alternation of haploid and diploid phases and the distinction between heterothallic and homothallic species.

After Mortimer and Hawthorne 1969.

distinguished five stages in the process (Figure 1.2). Meiosis in yeast shows considerable similarity with the same process in higher eukaryotes although the nuclear membrane does not break down during yeast meiosis.

Yeast cells and spores contain typical eukaryotic membrane systems including a nucleus, endoplasmic reticulum and mitochondria although normal mitochondria are absent in resting ascospores existing instead as small granules (Hashimoto et.al. 1958).

During stage IV (Figure 1.2) a prospore wall appears around each lobe of the four lobed nucleus. These prospore walls consist of a double membrane and finally join when the lobed nucleus separates to form the nuclei of the ascospores. Spore wall material becomes deposited between the two membranes. The spore wall differs antigenically from that of the vegetative cell (Snider and Miller 1966).

#### Biochemical events of sporulation.

It is only recently that the biochemical changes in sporulating cells have been examined in any depth. In Saccharomyces, however, very few biochemical markers have been established which are specific to sporulating cells. This contrasts with bacterial sporulation, notably in Bacillus, where numerous sequential biochemical events specific to sporulating cells have been found.

#### A. Changes specific to sporulating cells.

Pre-meiotic DNA synthesis is the best documented event specific to sporulation in yeast. The time at which this occurs is very variable due to differences in pre-sporulation culture conditions but generally it occurs fairly early in the sporulation process, a few hours after suspension in sporulation medium (Tingle et.al.

Figure 1.2 ( Moens and Rapport 1971)

Schematic representation of the nuclear events during meiosis and spore formation of Saccharomyces cerevisiae .

- a. Stage I. Spindle plaque has not duplicated and few microtubules are associated with it.
- b. Stage II. Spindle plaque has duplicated; more microtubules are observed.
- c. End of stage II; spindle plaques have migrated to face each other but have not yet begun to separate.
- d. Stage III. Spindle plaques are separating during the first meiotic division. Nucleus is elongating with its membrane intact.
- e. Beginning of stage IV. Spindle plaques have duplicated at the end of the first meiotic division.
- f. Stage IV. Spindle plaques have migrated to face each other for the second meiotic division.
- g. Stage IV. Second meiotic division with "prospore" wall shown. Spore wall formation beginning.
- h. Stage IV. Second division and spore wall formation are almost completed. Nuclear membrane is still intact.
- i. Stage V. Four spores are visible in the ascus. Spore maturation is in progress.

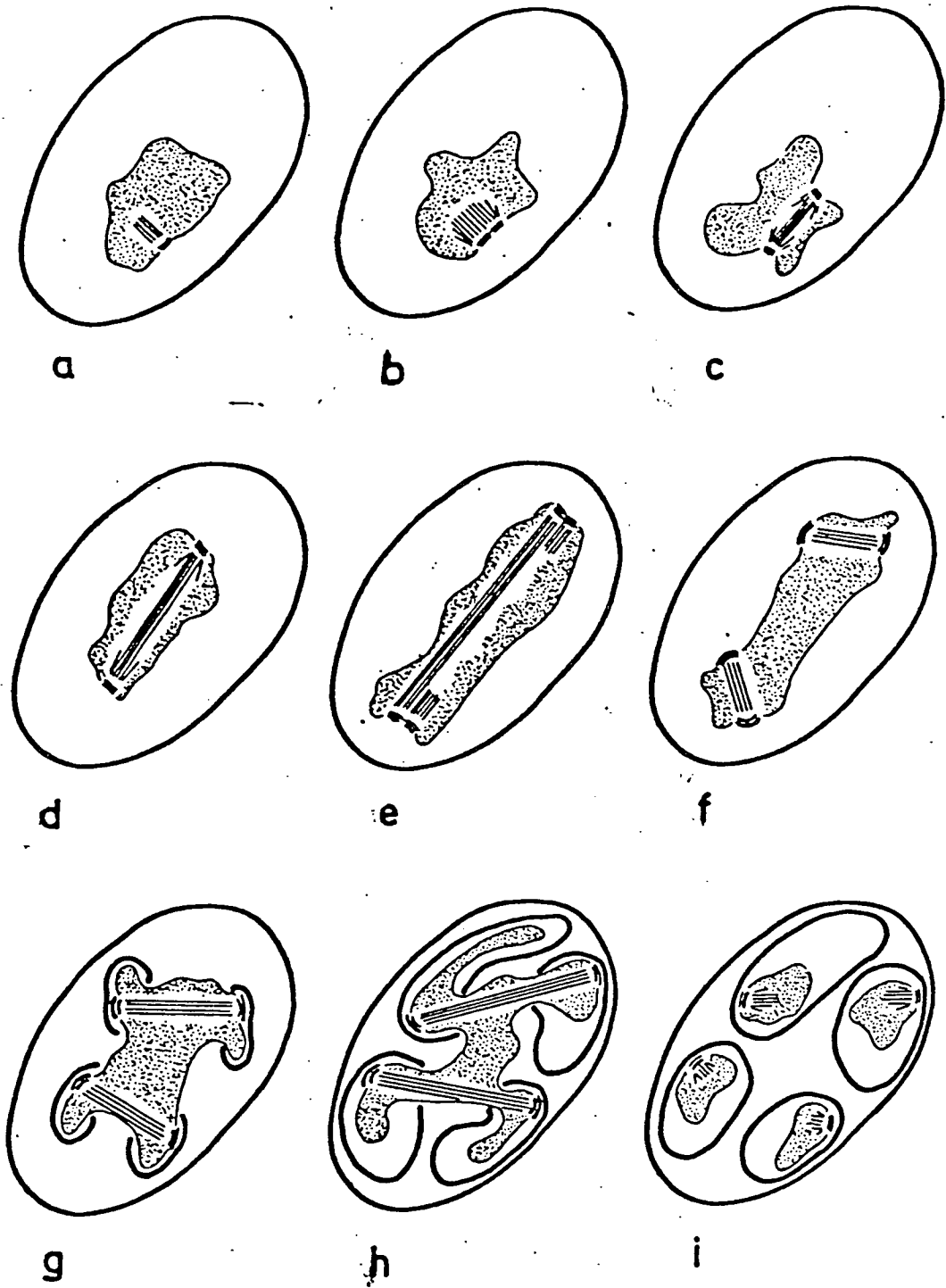


Figure 1.2

1973) and is evident as a doubling in DNA content (Croes, 1967, Esposito et.al. 1969).

The usual controls used in the study of events specific to sporulation include  $a/a$  or  $\alpha/\alpha$  diploids which under normal circumstances do not sporulate (Roman and Sands, 1953) nor undergo any of the events specific to sporulating cells such as pre-meiotic DNA synthesis (Roth and Lusnak, 1970), genetic recombination (Friis and Roman 1968) or nuclear division (Hopper et.al. 1974).

Meiotic recombination succeeds DNA synthesis (Roth, 1973). After cells are committed to intragenic and intergenic recombination commitment to chromosome disjunction and eventual formation of ascospores occurs. This point appears to coincide with the appearance of maximum numbers of recombinants (Esposito and Esposito 1974). Commitment, as is usually done in bacterial sporulation, is defined as the point at which cells in sporulation medium do not revert to vegetative growth when transferred to growth medium but go on to complete sporulation (Ganesan et.al. 1958). Commitment should be distinguished from "readiness" to sporulate. If cells in sporulation medium are transferred to water then sporulation will occur if these cells have acquired readiness. (Simchen et.al. 1972). Readiness is established before commitment and occurs quite early in the events of the sporulation sequence.

There has been a report of changes in the DNA-dependent RNA polymerase in yeast during sporulation (Magee 1974). A new peak of RNA polymerase activity was found to occur in  $a/\alpha$  sporulating diploids about eight hours after resuspension in sporulation medium. Peak Ib became very low. In  $\alpha/\alpha$  non-sporulating diploids this new peak was absent and peak Ib was present. The new peak in



a/ $\alpha$  cells was found to be partially sensitive to alpha amanitin. However, no further information is available as yet on this phenomenon and it does not appear to have been utilised as a marker event to date. Such experiments need to be interpreted with caution since similar changes in sporulating bacteria are induced by extracellular proteolytic enzymes during the preparation of extracts (Linn 1973). Indeed, more recently Klar et.al. (1976) have re-appraised these results in Saccharomyces and suggest that the new peak observed by Magee (1974) may similarly result from proteolytic modification of peak Ib during enzyme preparation as proteinases are abundant during yeast sporulation. Thus the concept of a sporulation specific alteration in RNA polymerase must as yet remain inconclusive.

Other established spore specific features occur rather late in the sporulation sequence. One is ether resistance (Dawes and Hardie 1974) of ascospores as compared to vegetative cells. Work is being carried out at present to determine at what stage the sporulating cells become significantly more resistant to ether. Since ether resistance is undoubtedly connected with maturation of the spore wall it may well serve in the future as a rapid method of testing for normal development of the ascospore wall. Ether resistance has already been successfully for enrichment of sporulated cells in a mixed culture (Dawes 1975).

Undoubtedly development of the spore wall involves the need for specific proteins which are not found in the vegetative cell. None as yet have been found although the spore wall has been shown to be antigenically different from the vegetative cell wall and this antigen is thought to be a protein (Snider and Miller, 1966).

However, this does not rule out the possibility that these new external proteins may normally be found inside the vegetative cell.

Glycogen degradation is the remaining spore specific event. Early in sporulation there is a rapid accumulation of glycogen, which is not specific to sporulating cells..This is followed by a period of degradation of soluble glycogen coincident with the maturation of the ascospores which appears to be specific to sporulating cells (Kane and Roth 1974, Hopper et.al. 1974). The extent of degradation, however, appears to be variable between different strains. Kane and Roth (1974) obtained only a 25% decrease in the total soluble glycogen whereas Hopper et.al. (1974) found a 75% decrease.

Therefore readily useable marker events for the progress of sporulation are relatively few in yeast. Hopper et.al. (1974) compared single dimensional polyacrylamide gel patterns of sporulating  $a/\alpha$  and non-sporulating diploid  $a/a$ ,  $\alpha/\alpha$  and found no differences in the protein banding patterns obtained under the same sporulation conditions but the patterns were different from those of vegetative cell proteins. While it would seem from the specific events discussed above that there must be proteins specific to sporulation these may be few in number and low in concentration and hence difficult to resolve with present techniques. It should also be noted that the method used by the above authors could only resolve a low proportion of the polypeptides present in the cells.

#### B Biochemical events non-specific to sporulating cells.

Many of the metabolic responses of diploids homozygous for

mating type a/a,  $\alpha/\alpha$  were similar to those of sporulating diploids a/ $\alpha$  on transfer to sporulation medium. Therefore it seems that many of the changes, although necessary to sporulating cells are also a reflection of a general adaption to altered nutrient conditions by all cell types whether spore formers or not.

As mentioned above a number of other metabolic changes occur throughout sporulation. Pulse labelling of sporulating cells showed that RNA synthesis proceeds continuously during sporulation with a peak of maximal synthesis early in the process and another peak towards the end (Esposito et.al. 1970). RNA content, however, after an initial rise declines steadily (Esposito et.al. 1969, Sands and Miyake 1971). Most of the RNA is ribosomal as in vegetative cells but it has not yet been determined if new ribosomal RNA synthesis is really necessary for sporulation.

There is an increase in protease activity at the onset of sporulation (Betz and Weiser 1976). The decrease of insoluble radioactivity in pre-labelled vegetative cells has been used as a measure of protein turnover (Esposito et.al. 1969, Hopper et.al. 1974). 30-50% of the vegetative cell protein is degraded. Protein synthesis is required throughout sporulation (Magee and Hopper 1974) since cycloheximide inhibits the process at all stages. As sporulation occurs in the absence of an exogenous nitrogen source the internal amino acid pool and those released from protein turnover must be sufficient for protein synthesis (Esposito et.al. 1969, Halvorson 1958). In contrast most of the carbon and energy requirements for sporulation are derived from exogenous carbon sources such as acetate (Esposito et.al. 1969). This need

for an exogenous carbon source is illustrated by the inability of either logarithmically growing or stationary phase cells from acetate medium to sporulate when transferred directly to distilled water (Simchen et.al 1972).

#### Mitochondrial requirement of sporulation.

Mitochondrial activity, and under certain circumstances mitochondrial protein synthesis, are essential for sporulation. Respiratory deficient strains (petites) are unable to sporulate wheter the mutation is nuclear or cytoplasmic (Ephrussi 1953, Sherman 1963).

In Bacillus also, increases in TCA cycle enzymes have been observed at the onset of sporulation (Hansen et.al. 1970) similar to that in yeast (Miyake et.al. 1971). Mutational loss of any of the TCA cycle enzymes in Bacillus leads to a block in sporulation at  $t_0$  or  $t_1$  (Fortnagel and Freese 1968). It is proposed that these mutants cannot maintain the ATP concentration necessary for sporulation (Freese 1972, Ohné and Rutberg 1976). This is supported by the observation that a malate dehydrogenase mutant sporulates normally when supplied with oxaloacetate (Ohné<sup>and Rutberg</sup> 1976). Cytochromes are induced in Bacillus early in sporulation and mutants lacking cytochromes do not sporulate (Taber et.al. 1972). In view of the fact that petite mutants of yeast do not sporulate it appears that Bacillus and yeast have similar requirements in this respect.

Puglisi and Zennaroe (1971) demonstrated an inhibition of sporulation when erythromycin-sensitive strains were challenged with erythromycin, an inhibitor of mitochondrial protein synthesis. However, more interestingly there seems to be no requirement

for the mitochondrial genome per se for successful sporulation provided cells are pre-grown in a medium in which they are already adapted to oxidative metabolism. Kuenzi et.al (1974) in an elegant series of experiments showed that if logarithmically growing cells in acetate medium were subjected to ethidium bromide treatment in the last generation before transfer to sporulation medium sporulation proceeded as normal, the sporulation frequency being only slightly less than that of the untreated culture. However, the spores derived from this regime were phenotypically petite and failed to grow on non-fermentable carbon sources. When late log glucose grown cells were treated in the same way prior to transfer to sporulation medium sporulation was completely absent. These cells from late log glucose grown cultures are not fully adapted to oxidative metabolism hence there is still a requirement for mitochondrial protein synthesis during sporulation. It follows from this that mitochondrially synthesised proteins must have considerable stability.

#### Utilisation of acetate during sporulation.

Examination of the incorporation of acetate in S.cerevisiae indicates that it is an important source of metabolites for biosynthetic reactions which occur during sporulation. When ascus development was complete 62% of the acetate taken up was found to have been metabolised to  $\text{CO}_2$ , 22% was in the soluble pool and 16% had been incorporated into macromolecules (Esposito et.al. 1969). It seems that utilisation of acetate for sporulation must involve the glyoxylate cycle in order to ensure a sufficient supply of gluconeogenic precursors. There is, however, some controversy and perhaps misunderstanding of the functioning of the

glyoxylate cycle during sporulation. Croes (1967) proposed that sporulation may be triggered by an insufficiency of the cycle. This does not, however, mean that the glyoxalate cycle does not function but merely that its output, possibly its maximum output, is insufficient to meet both the energetic and biosynthetic demands of the cell in the absence of a nitrogen source. Betz and Weiser (1976) found that the levels of isocitrate lyase, one of the glyoxalate cycle enzymes, was high at the onset of sporulation but declined steadily thereafter.

Control mechanisms involved in the regulation of yeast sporulation.

There are two different stages of control; control at the level of initiation, which is the main consideration of this thesis, and the subsequent control of the sporulation sequence. The existence of two different stages of control does not necessarily mean that they are regulated completely independently of each other. For example subsequent regulation of sporulation is sensitive to nutritional control just as is initiation. Whether this acts at the same or different levels in each case is undetermined.

In establishing sequence it appeared that cells became committed to undergo recombination before becoming committed to undergoing meiotic division (Esposito and Esposito 1974). Haber and coworkers (1975) maintain that premeiotic DNA synthesis and commitment to recombination are co-ordinately regulated; all mutants studied that were defective in pre-meiotic DNA synthesis were also defective in recombination. On the basis of his results, however, Simchen and Hirschberg (1977) suggest that commitment to recombination does not depend on the completion of pre-meiotic DNA synthesis.

Certain diploid strains of Saccharomyces which produce exclusively two-spored asci with diploid spores have been examined by electron microscopy during sporulation. It appears that the first meiotic division is suppressed and one equational division results in two diploid spores (Moens 1974). No genetic data has been presented as yet to support this conclusion but Haber et.al. (1975) suggest that these strains indicate that ascospore formation is separable from typical meiosis. However, mutants defective in conventional intergenic recombination yet

capable of sporulation have been sought but not yet found (Haber et.al. 1975).

Therefore a study of sequence regulation and dependency events of the sporulation process using appropriate mutants is an essential to an understanding of all aspects of sporulation control.

#### Control of initiation of sporulation.

Initiation of sporulation is subject to three forms of control:

- i. Nutritional, both carbon and nitrogen source.
- ii. Cell-cycle dependency.
- iii. Mating type locus.

This section is devoted to a consideration of these three levels of control and how they could influence sporulation.

#### Carbon source control.

Miller (1957) observed that addition of readily metabolised carbon sources, glucose, fructose and mannose, produced a severe inhibition of sporulation at fairly low concentrations (0.05-1.0%). Above 0.5% hexose complete inhibition of sporulation was observed in sporulation medium. This effect was also found using galactose but the inhibition produced was not as strong. Fast (1973) found that transfer of cells during exponential growth on glucose to acetate sporulation medium gave no sporulation. In contrast transfer of early stationary phase cells from glucose medium gave a high frequency of spore formation. Croes (1967) demonstrated a switch at the end of log phase on glucose from fermentative to oxidative metabolism and a concomitant utilisation of ethanol accumulated during growth on glucose. Esposito et.al. (1969) found no lag in the ability of late logarithmic- early stationary phase glucose grown cells to oxidise acetate. Hence there is



ample evidence for a repression of sporulation by readily utilised hexose sugars.

The similarities between carbon repression of sporulation and carbon catabolite repression of the synthesis of inducible enzymes are striking and the question arises as to whether the two are under separate similar or identical control(s). Carbon catabolite repression is the process whereby cells do not synthesise enzymes for breakdown of an energetically less favourable carbon source in the presence of one that is more readily utilised such as glucose. In yeast there are many enzymes which are subject to catabolite repression by glucose. Certainly many of the metabolic shifts that occur early in sporulation and which are necessary such as the complete derepression of the TCA cycle and respiratory enzymes are also subject to glucose repression under vegetative growth conditions. Therefore it is difficult to distinguish the possibility that there is a direct effect of a rich carbon source on sporulation (i.e. a control mechanism which is unique to sporulation) from the same mechanism (i.e. catabolite repression) as that acting on enzyme synthesis in vegetative cells. If the latter were true glucose inhibition of sporulation would merely be due to an inhibitory effect on essential sporulation functions. There have been few genetic studies on the carbon control of sporulation in yeast, most of the work has been mainly of a physiological nature. Therefore most of the information on control of carbon repressible systems comes from studies, both biochemical and genetical, on carbon catabolite repression of enzyme synthesis. This topic will be examined in a later section as knowledge of progress in

in this field is relevant to the control of sporulation.

The search for mutants insensitive to carbon repression of sporulation (hyper-repressed mutants would be difficult to recognise owing to the pleiotropic nature of spo mutants) may provide considerable information on carbon catabolite control of all such repressible systems. But, as sporulation is sensitive to repression by glucose for a considerable length of time during the sequence (Tingle et.al. 1973), it may be that there are several sporulation genes which are individually sensitive to carbon repression and as such, single control mutations may not be isolatable.

However, in yeast glucose repression through the course of sporulation may only reflect the length of time that sensitive vegetative enzyme systems are required during sporulation and, provided that all the necessary enzymes are under the same catabolite control system, it should be possible to isolate mutants which sporulate in the presence of glucose.

Mutants of Bacillus subtilis have been reported which sporulate in the presence of glucose or on an amino acid mixture (Ito and Spizien 1973). One type of mutant, designated as cata exhibited excess production of exoprotease. Growth of the mutant on glucose was normal and histidase production remained subject to normal glucose repression. Ito and Spizien concluded that there was no defect in general catabolite repression. However, sporulation in Bacillus can occur if either carbon or nitrogen repression is relieved which may preclude any common catabolite control between sporulation and enzyme synthesis.

The B.subtilis sacU<sup>h</sup> mutants isolated by Kunst et.al. (1974)

exhibit excess production of extracellular sucrase, exoprotease and the ability to sporulate in the presence of glucose or an amino acid mixture. This phenotype is the result of a single mutation, separate from the cata gene (Lepesant-Kejzlarova et.al. 1975).

Coote (1974) has attempted to determine if the catabolite repression of inducible enzyme synthesis and carbon repression of sporulation in Bacillus subtilis are controlled by the same mechanism but the results were inconclusive.

Carbon catabolite repression of enzyme synthesis in bacteria is believed to be mediated by cyclic 3',5' -adenosine monophosphate (cAMP) and the cAMP receptor protein (Pastan and Perlman, 1970, de Crombrugne et.al. 1971). The mechanism of control at the level of cAMP will be considered in relation to catabolite repression of enzyme synthesis discussed in a later section.

It is a reasonable assumption that the carbon control of sporulation may be regulated by the cAMP system but no adenyl cyclase has been found in Bacillus subtilis (Ide 1971, Setlow 1973) and there appears to be no information on changes in cAMP levels in vegetative and sporulating cells.

Studies on the regulatory effects of cAMP in yeast are preliminary by comparison but evidence for its involvement in catabolite repression of enzyme synthesis is accumulating.

In sporulating cells the period of glucose sensitivity may reflect a period of control by cAMP. Tsuboi et.al. (1972) reported repression of sporulation in S.cerevisiae by glucose was reversed by addition of  $10^{-5}$ - $10^{-4}$  M cAMP to the sporulation medium. Peaks in the levels of cAMP have been observed to appear during the glucose sensitive period of sporulation (Hartig and Breitenbach 1977).

Addition of 0.2% glucose to the sporulation medium both prevented the appearance of these peaks and sporulation thus demonstrating a relationship between intracellular cAMP and glucose repression of sporulation. Watson and Berry (1977) also observed a pattern of peaks in cAMP levels during the early stages of sporogenesis. These peaks were greater in cells pre-grown on 2% glucose than in cells pre-grown on 5% glucose.

Evidence is therefore accumulating for a possible regulatory rôle of cAMP during yeast sporulation. Again, however, there are no data to indicate whether the requirement is specific to control sporulation or as a positive regulator of transcription of genes (such as TCA cycle loci) which are necessary but not specific for sporulation. In addition, it might be unwise to assume that the cAMP mechanism is the only positive control system involved in release of glucose repression in higher cells.

#### Regulation by nitrogen source.

Sporulation in yeast is routinely induced by transferring the cells to a nitrogen free medium with acetate as carbon source (Esposito et.al. 1969, Fowell 1969). Miller (1963) obtained inhibition of sporulation with a variety of nitrogen compounds when added to the acetate sporulation medium. These included most amino acids and ammonium ion. The severity of the inhibition varied according to the nature of the nitrogen source. Yeast extract has also been found to be inhibitory to sporulation in the presence of acetate (Haber and Halvorson 1975).

Nitrogen repression of sporulation in yeast has received little attention to date. Bacillus mutants have been isolated in which sporulation shows a reduced sensitivity to repression by amino

acids but these single gene mutants also show insensitivity to glucose (see preceding section on carbon repression).

As with carbon repression the study of nitrogen repression has mainly been directed towards an understanding of the control of synthesis of enzymes subject to repression by nitrogen sources, such as ammonium ion. There are several examples of such enzymes in yeast, and again the similarity between regulation of synthesis of these enzymes and the regulation of sporulation by readily utilisable nitrogen sources is striking. There has been considerable controversy regarding the mediator of repression of enzyme synthesis: ammonium ion and amino acids both appear to be capable of producing repression dependent on the circumstances. It appears that a similar situation may exist in sporulation as Miller (1963) obtained inhibition by both ammonium ion and amino acids. The problem has, up to the present, not been subject to further investigation. The merits of ammonium ion and amino acids as repressors of enzyme synthesis, together with an appraisal of the considerable advances made recently in the understanding of the genetic control of nitrogen repressible enzyme synthesis is presented in a later section.

A regulatory rôle of glutamine synthetase in regulation of nitrogen repressible enzyme synthesis, distinct from its catalytic function, has been proposed in Klebsiella aerogenes (Prival et.al. 1973). This is also described in the section on nitrogen regulation of enzyme synthesis. In Bacillus megaterium a similar rôle of glutamine synthetase in the regulation of sporulation has been postulated (Reysset and Aubert 1975). They base this hypothesis on the observation that most mutants defective in glutamine

synthetase also show poor sporulation and that these two characters always undergo co-reversion. In particular they isolated one mutant, apparently quite rare, which exhibited no enzymatic activity and only low recognition by antibody directed against glutamine synthetase (wild-type enzyme) but which possessed normal ability to sporulate. They interpret this mutation as affecting only the enzymatic properties of the protein which would be a fairly rare event. On the basis of these observations and others (Elmerich and Aubert 1975) they propose that glutamine synthetase may be the receptor of a low molecular weight effector from the early part of the purine biosynthetic pathway and that this complex acts in the regulation of sporulation. There is, however, no evidence to implicate glutamine synthetase as a mediator in yeast sporulation.

Work on nitrogen repression of enzyme synthesis has led to the finding that there is an interaction between carbon and nitrogen repression. For example, carbon catabolite repression has been found to be a prerequisite for ammonium ion repression of proline oxidase and acetamidase (Arst and Cove 1973) but this may not be the case with ammonium ion repression of other enzymes such as arginase or asparaginase. The authors also state that especially where both carbon and nitrogen repression are involved in regulation of a particular system it may be difficult to distinguish between the two forms of repression. As sporulation in yeast is subject to repression by both carbon and nitrogen sources this difficulty may also be apparent. This is considered further in the results section but it is perhaps worth emphasising that derepressed sporulation mutants of Bacillus (mentioned above)

exhibit their phenotype in the presence of either glucose or amino acids, thus it cannot be determined whether they are altered in either carbon or nitrogen repression or a mechanism common to both.

#### Cell cycle control of sporulation.

There is little information available on the differences between mitosis and meiosis and the regulatory events that determine which will be initiated. Recessive mutations have been isolated in S. cerevisiae and S. pombe which allowed normal growth and mitosis but do not permit meiosis (Esposito and Esposito 1969, Bresch et.al. 1968). This suggested that the two different forms of nuclear division might be, at least in part, under separate genetic control (Esposito et.al. 1969). A diagrammatic representation of the cell cycle of S. cerevisiae is presented in Figure 1.3.

More recently Simchen (1974) has tested certain genes essential to the mitotic cell cycle for their requirement in meiosis. These genes are represented by established temperature sensitive mutations affecting the cell division cycle (cdc) as described in a review by Hartwell (1974). Of the twenty cdc mutants tested thirteen appeared essential for meiosis and sporulation because diploid cells homozygous for these mutations were severely impaired in their spore forming abilities at the restrictive temperature. This included mutants classified by their inability to undergo spindle plaque duplication, initiation of DNA synthesis, DNA synthesis and nuclear division in the mitotic cell cycle. However, the remaining seven genes did not appear essential for meiosis. These included cdc24 (defective in bud emergence) and cdc3, 10, and 11 (defective in cytokinesis).

Notably these mitotic processes are not a morphological part of meiosis, separation of cytoplasm occurring by a very different process during meiosis (Figure 1.2). Simchen<sup>and Hirschberg</sup> (1977) has made a more detailed study of one of the genes, cdc4, required for both meiosis and mitosis. Mutation in this gene appears to block the initiation of nuclear DNA replication and the separation of the replicated spindle plaques (Hartwell 1974). Cells arrested at the restrictive temperature of 34° during meiosis show a high degree of commitment to recombination but very reduced DNA synthesis. It is suggested that commitment to recombination does not depend on the completion of pre-meiotic DNA synthesis. Shifts to the restrictive temperature at different points throughout the sporulation indicated that the cdc4 function is necessary at several points during meiosis i.e. after initiation of DNA synthesis. Therefore it seems that the organisation of meiosis may be more complex than that of mitosis.

There is increasing evidence indicating that cells may enter the sporulation process from G<sub>1</sub> of the cell cycle (Figure 1.3), between cytokinesis/cell separation and the initiation of DNA synthesis. In the mammalian cell cycle the initiation of differentiation occurs during G<sub>1</sub> (Vendrely and Vendrely 1956). In synchronised yeast it has been demonstrated that stationary phase cells accumulate in G<sub>1</sub> as unbudded cells arrested prior to DNA synthesis (Williamson and Scopes, 1962, Williamson 1964). Stationary phase cells can enter meiosis without the intervention of a mitotic division indicating that cells can enter meiosis from G<sub>1</sub> (Croes 1966).

Examination of cells after a switch from a growth to a



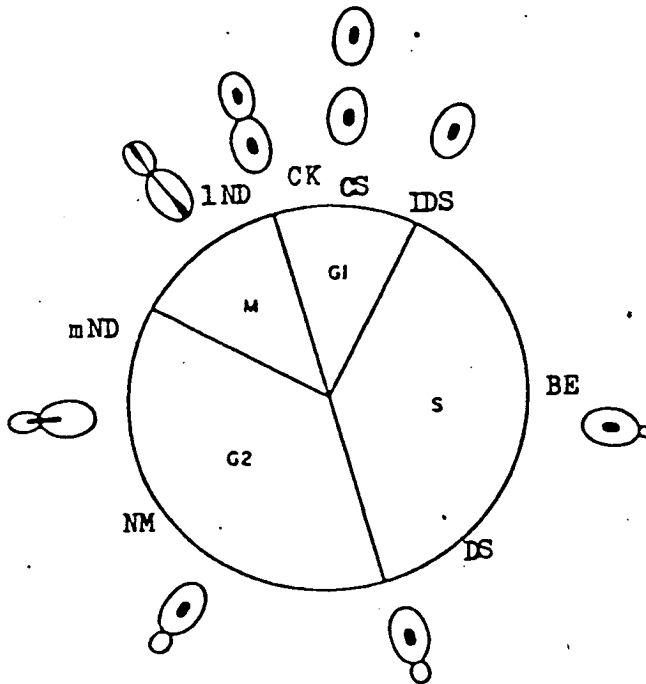


Figure 1.3

The sequence of events in the cell division cycle of yeast:  
 iDS, initiation of DNA synthesis; BE, bud emergence; DS, DNA  
 synthesis; NM, nuclear migration; mND, median nuclear division  
 1ND, late nuclear division; CK, cytokinesis; CS, cell separation.  
 Other abbreviations;  $G_1$ , time interval between previous cytokinesis  
 and initiation of DNA synthesis; S, period of DNA synthesis;  
 $G_2$ , time between DNA synthesis and onset of mitosis; and M, the  
 period of mitosis. After Hartwell *et.al.* 1974.

sporulation medium has prompted the proposal that cells which have passed the  $G_1$  phase of the cell cycle go on to complete bud emergence, cytokinesis and cell separation. That is they complete the mitotic division cycle, whereas unbudded cells in  $G_1$  proceed directly to meiosis (Milne 1972).

Therefore initiation of meiosis and spore formation may depend on an event which occurs during the  $G_1$  of the cell cycle. This concept will be considered in the discussion in relation to the sporulation mutants described in this work.

#### Mating type control of sporulation.

Only diploids or higher ploidy yeast which are heterozygous for mating type (  $a/\alpha$  or  $a/a/\alpha/\alpha$  etc) are capable of sporulation. Diploids which are homozygous at the mating type locus ( $a/a$  or  $\alpha/\alpha$ ) do not normally undergo premeiotic DNA synthesis or sporulation (Roman and Sands 1953, Roth and Lusnak 1970). Therefore satisfaction of the mating type requirement is a necessary pre-requisite for sporulation and is expressed at least once very early in the sporulation sequence probably at premeiotic DNA replication.

There has been a fairly recent advance in the study of mating-type control. Mutants have been isolated in which premeiotic DNA synthesis, and to some extent sporulation, are uncoupled from the mating type control (Hopper and Hall 1975). This mutation, if present in  $a/a$  or  $\alpha/\alpha$  diploids allows all the cells in the population to carry out premeiotic DNA synthesis: only 20% of the cells go on to complete sporulation. Recombination occurred only in the spore forming cells.

This SP1 mutant (as it is designated) is dominant and it is suggested that its effect is to uncouple pre-meiotic DNA synthesis but not meiotic recombination from mating type control. Consequently

Hopper and Hall (1975) further propose that the mating type control is exerted at more than one point in the sporulation sequence.

## Regulation of enzyme synthesis by carbon and nitrogen repression.

### Carbon catabolite repression.

This has been extensively studied in bacterial systems in for example the lactose and arabinose operons (see Pastan and Adhya 1976, Gonzalez and Shepherd 1977 for reviews of regulation).

Briefly, control is exerted at two levels:

- 1) At the level of induction, a repressor protein binds to an operator gene tightly linked to the structural genes for the enzymes and prevents binding of the RNA polymerase. The inducer, for example lactose, has a higher affinity for the repressor protein and thus if present, prevents its binding to the operator. This is an example of negative control.
- 2) Catabolite repression mediated by the cAMP receptor protein (CRP). cAMP regulates transcription of the structural genes of the operon by binding at the promoter site of the DNA adjacent to the operator and increases the frequency of initiation. This is an example of positive control. One of the mechanisms of glucose action is to lower the internal concentration of cAMP.

In yeast there are many enzymes which are subject to catabolite repression by glucose but analysis of control is still in its early days and the mechanism may prove to be different from or more complex than that in bacteria. The synthesis of invertase (Lampen 1968), maltase and the maltase uptake system (van Wijk et.al. 1969), respiratory enzymes (Strittmatter 1957, Polakis and Bartley 1965) are all subject to repression in the presence of glucose.

There have been isolated in S. cerevisiae mutants in which the enzymes of specific pathways do not respond to catabolite repression.

For example Ciriacy (1976) has obtained mutants in which the synthesis of alcohol dehydrogenase II was insensitive to glucose repression. Certain mutations in the regulatory gene MAL2 for maltase synthesis cause a glucose resistant constitutive synthesis of this enzyme (Zimmermann and Eaton 1974). Montencourt et.al.(1973) have isolated a mutant with hexose resistant invertase synthesis but this mutant was found not to be amenable to genetic study.

It was not however until recently that mutants have been isolated which are altered in the glucose repression of several functions as the result of a single mutation. In S.cerevisiae Zimmermann et.al.(1977) selected for and obtained a single gene mutant which had a pleiotropic inability to utilise glycerol and maltose, i.e. was permanently repressed for several catabolite repressible functions. This mutation was recessive and designated cat1-1 (wild-type CAT1). It did not, however, prevent fermentation of sucrose or raffinose. This contrasts with the phenotypes of mutants lacking adenyl cyclase or cAMP receptor protein in E.coli (Perlman and Pastan 1969, Zubray et.al.1970, Emmer et.al.1970). These have a pleiotropic effect on growth on all carbon sources apart from glucose i.e. all carbon sources requiring cAMP for synthesis of the enzymes concerned with their metabolism.

cat1-1 strains were observed not to undergo derepression of maltase, fructose 1,6-diphosphatase and isocitrate lyase on shifting cells from glucose to ethanol. Derepression of the synthesis of malate dehydrogenase was considerably retarded though this did occur to some extent.

This mutant was used to select for revertants, not to the wild type, but to a form which exhibited a reduced sensitivity to the

carbon catabolite control. Two revertants were obtained with this phenotype. One, cat2-1, was a recessive external suppressor of cat1-1. The other, cat1-2<sup>d</sup>, was dominant and allelic to cat1-1. Both mutations were initially seen to cause an earlier derepression of the above mentioned enzymes than was found in the wild type on transfer from glucose to ethanol medium. The fully repressed or derepressed levels were not altered. Recent further studies (F,K, Zimmermann pers.comm.) revealed that fully derepressed levels of these enzymes are insensitive to the addition of 2% glucose.

Based on these mutant characteristics Zimmermann et.al. (1977) proposed a model for regulation of derepression of carbon catabolite sensitive enzymes: after catabolite repression is relieved (on removal of glucose) gene CAT1 becomes active and prevents the product of CAT2 from functioning as a repressor of enzyme synthesis. This CAT2 gene product must be removed before derepression can occur. The time required for this causes a delay in derepression after glucose is depleted. The cat1-1 gene product cannot block CAT2 action hence no derepression can occur. The product of the cat2-1 gene is inactive in repression and thus derepression can occur after catabolite repression has been removed. The cat1-2<sup>d</sup> mutant gene is permanently active as a repressor of CAT2 and hence eliminates the delay in derepression. It seems that this hypothesis alone does not fully explain the phenotypes of the described mutants and the possibility must be considered that there exists another control mechanism which acts in conjunction with the proposed control.

Whatever the outcome of further study these mutants certainly

show considerable potential for analysing the mechanisms of carbon catabolite repression in yeast. Preliminary studies are being carried out in this laboratory to determine the effect of these mutations on sporulation, alone, and in conjunction with other likely control mutations. It seems likely that these catabolite insensitive mutants may well prove helpful in determining the nature of carbon control of sporulation, specific or not.

Bailey and Arst (1975) have also reported the isolation of mutants affecting regulation of glucose repressible functions in Aspergillus.

Studies on the regulatory effects of cAMP in yeast are preliminary by comparison but evidence of its involvement in catabolite repression is accumulating. Lower concentrations of cAMP were found in S.carlsbergensis under conditions of catabolite repression by 2% glucose. During adaption to maltose there was an eight fold increase in the cAMP levels (van Wijk and Konijn 1971). Wiseman and Lim (1974) observed a slow induction of  $\alpha$ -glucosidase (maltase) activity by 10mM cAMP in the presence of 2% glucose and 4% maltose in stabilised yeast protoplasts. They found no similar effect using ATP, 5'AMP, 3'AMP or cyclic adenosine 2',3'-mono phosphate. A possible involvement of cAMP in respiratory adaption in S.cerevisiae is indicated by its ability to overcome the severe inhibition of 10% glucose on respiratory adaptation again using protoplasts (Fang and Butow 1970). However, the effect was found not to be specific for cAMP.

### Regulation of nitrogen repressible enzyme synthesis.

In Aspergillus and Saccharomyces there are a range of enzymes subject to nitrogen repression of their synthesis. In yeast these include arginase,  $\text{NAD}^+$ -dependent glutamate dehydrogenase, allantoinase and urea amidolyase (Dubois et.al.1973) while in Aspergillus more unusual enzymes such as nitrate reductase xanthine dehydrogenase and urate oxidase are also included (Arst and Cove 1973). In addition to being subject to control by nitrogen repression these enzymes are in general subject to control by induction (Wiame 1971, Arst and Cove 1973).

The nitrogen regulation of these enzymes is complex. Most authors agree that ammonium ion is the main effector of repression (Arst and Cove 1973, Dubois et.al.1973) but recent reports suggest that glutamine and asparagine may be as efficient as signals for repression of some enzymes as ammonium ion. (Hynes 1974, Dubois, et.al.1977). It is difficult to identify the primary signal due to the rapid interconvertibility of glutamine and ammonium ion. However, as allantoinase is subject to greater repression by glutamine than ammonium ion in S.cerevisiae, a form of repression mediated by glutamine is indicated. Bossinger et.al.(1974), however, reported only a very small repressive effect of ammonium ion on allantoinase and arginase when added to cultures utilising allantoin or arginine as sole nitrogen source. These ammonium ion effects were minor compared to the severe inhibition exerted by readily utilised, in particular transaminatable, amino acids such as serine, glutamine and asparagine. The authors further suggest that the repression observed with ammonium ion may be due to its metabolism to transaminatable amino acids within the cell. Dubois



et.al.(1977) now accept that repression occurs in cells growing in either ammonium ion, glutamine or asparagine but dispute the proposal that ammonium ion does not act as a direct mediator in repression. The evidence for this comes from mutants (designated NADP<sup>+</sup>-gdh) lacking the anabolic NADP<sup>+</sup>-dependent glutamate dehydrogenase. This defect leads to reduced growth on minimal medium with ammonium ion as nitrogen source due to poor assimilation of nitrogen by the mutants (Grenson and Hou, 1972). The mutation also causes a derepression of several ammonium ion repressible activities including arginase, urea amidolyase, allantoinase and several permease functions. Addition of glutamate restores normal growth rate presumably because the block on nitrogen assimilation has been passed, but repressed levels of arginase are not restored in an operator constitutive mutant (Dubois et.al.1974). On the basis of this evidence a scheme has been proposed whereby the signal, ammonium ion, is received by the NADP<sup>+</sup>-dependent glutamate dehydrogenase and this modified complex acts together with another effector(s) to mediate repression possibly at the level of the gene (Dubois et.al.1977). There is evidence to support the possibility that induction and repression of nitrogen repressible enzyme synthesis occurs at the level of transcription both in yeast (Lawther and Cooper 1975) and in Aspergillus nidulans (Bartnik and Weglenski 1973).

However, Bossinger and Cooper (1975) remain in disagreement with this proposed "direct" effector rôle of the NADP<sup>+</sup>-GDHase although (1973) Pateman et.al. have proposed a similar regulatory function of the enzyme in Aspergillus.

Another mode of nitrogen control proposed by Dubois et.al.(1977)

involves glutamine as a metabolic signal received by the product of a gene gnrR with the possible participation of glutamine synthetase to effect repression of certain enzymes (excluding arginase which appears to be controlled by the ammonium ion/ $\text{NADP}^+$ -GDHase system). In addition they have identified a product from the gene gdhCR which appears to be a common regulatory element in nitrogen catabolite repression.

Although the regulatory rôle of glutamine synthetase in yeast is not clear the system has been extensively investigated in Klebsiella aerogenes (Prival et.al.1973). Here mutants exhibiting constitutive synthesis of glutamine synthetase produce histidase and proline oxidase even when supplied with excess ammonium ion despite the fact that the high levels of glutamine synthetase are present in the adenylated form which is inactive physiologically. The reverse is also true: putative lesions in the structural gene for glutamine synthetase do not show production of histidase when nitrogen catabolite repression is relieved.

Although these mutants altered in aspects of nitrogen metabolism seem likely to be involved in nitrogen repression it has not been established that the mutation in question has directly altered the regulatory elements mediating ammonium ion repression. Indeed as these mutations, with the possible exception of the gdhCR gene product, individually affect only certain nitrogen repressible activities it seems likely that there exists a regulatory element common to all aspects of nitrogen repression. In yeast this may be the gdhCR gene product but the possibility hasnot yet been fully investigated.

Studies on such a regulatory locus in Aspergillus have been

presented by Arst and Cove (1973). Certain lesions in a locus designated areA lead to a pleiotropic loss of the ability to use nitrogen sources other than ammonium ion(areA<sup>r</sup>). This is paralleled by low levels of ammonium ion repressible enzymes. The phenotypes of these areA<sup>r</sup> alleles are very variable. They can be temperature sensitive on some nitrogen sources but not on others and the nitrogen sources on which temperature sensitivity occurs are different for different alleles. At the areA locus certain mutations lead to derepression of one or more ammonium ion repressible activities (areA<sup>d</sup>) but do not affect the control of synthesis by induction. Again the phenotypes of areA<sup>d</sup> alleles are variable. The areA<sup>r</sup> (hyper-repressed) mutations are epistatic to the NADP<sup>+</sup>-gdh mutations mentioned previously.

The areA locus is defined by these authors as specifying a protein which is required for the synthesis of nitrogen repressible enzymes. The areA product is either absent or inhibited in its action in the presence of ammonium ion i.e. a positive control system. This hypothesis is supported by genetic analysis of the areA mutants. areA<sup>r</sup> alleles appear to be recessive to the wild type whereas areA<sup>d</sup> alleles appear to be semi-dominant to the wild-type.

More recently partially repressed mutants have been isolated at another locus in Aspergillus designated tamA (Kinghorn and Pateman 1975). These tamA<sup>r</sup> mutants are like those at the areA site epistatic to the NADP<sup>+</sup>-gdh mutations and also to the areA<sup>d</sup> alleles which produce a derepressed phenotype. This suggests that the tamA locus is also directly involved in nitrogen repression and further studies should clarify this.

### Sporulation derepressed strains in the study of sporulation.

Genetic studies of the control of sporulation have in some directions been hampered by the pleiotropic nature of asporogenous (spo) mutants. spo mutants do not express any of the developmental events past the point of the block and therefore mutants blocked early in the sequence are difficult to identify as control mutants or initiation mutants.

It has previously been established that sporulation in Saccharomyces can only occur when both carbon and nitrogen source repression are relieved (Miller 1963). Therefore mutants which sporulate in the presence of a repressing carbon or nitrogen source should provide useful tools in the study of the regulation of yeast sporulation as these are likely to be control mutants.

In this laboratory mutants have been isolated which sporulate extensively under nutrient conditions where the wild-type does not. These have been designated spd (sporulation derepressed) mutants (Dawes, 1975).

These spd mutants were isolated due to their ability to sporulate on a complete medium. Preliminary studies (Dawes 1975) demonstrated that an spd1 mutant ID16D underwent only restricted growth on YEP-glycerol, a rich medium containing yeast extract and peptone as nitrogen source with glycerol as carbon source. Asci began to appear after 20-30 hours in YEP-glycerol (Figure 1.5). As sporulation of the mutant and wild-type strains normally takes about 20-24 hours under most favourable conditions, a percentage of the mutant population must have initiated sporulation soon after transfer to YEP-glycerol. Asci appeared in the wild-type only 20 hours after stationary phase was reached.

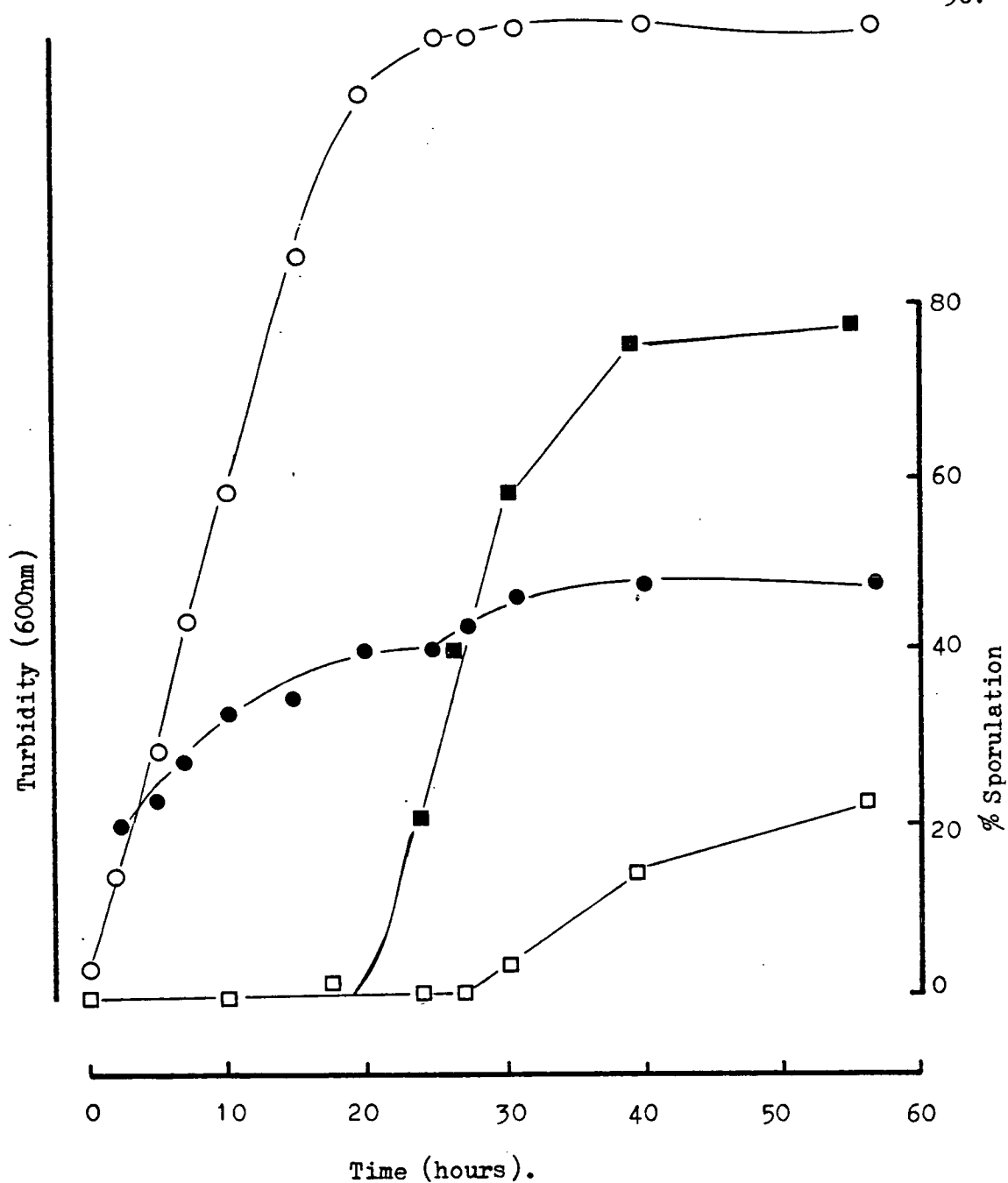


Figure 1.5

Growth and sporulation of wild-type and derepressed mutant ID16D on a five-fold dilution of YEP-glycerol medium.

( From Dawes 1975)

- growth of S41; ● growth of ID16D; □ sporulation of S41;  
 ■ sporulation of ID16D.

Another characteristic of the spd1 mutants is their capacity to sporulate heavily on a rich glucose medium (YEPD) after stationary phase is reached. This is shown in Figure 1.4 for ID16D (Dawes 1975). The wild-type over this 60 hour time course showed no sporulation.

ID16D also sporulated after growth on glucose minimal medium when the glucose concentration was low but ammonium ion concentration fairly high but not when the glucose concentration was high and the ammonium ion concentration low (Dawes 1975).

From these results it was proposed that the spd mutants might have become insensitive to the nitrogen control of sporulation such that when the carbon source was poor or limiting i.e. carbon control relieved, sporulation occurred despite the presence of a repressing nitrogen source.

Examination of this hypothesis has been approached in several ways. Studies have been carried out to establish a more complete picture of the phenotype(s) of the spd1 mutants as compared to the wild-type. For example, to determine if the poor growth/ derepressed sporulation phenotype observed on glycerol also extends to other carbon sources. Such an analysis may provide clues to any possible biochemical defect.

Transport and incorporation studies of  $^{14}\text{C}$  labelled substrates on which the spd mutants do not grow are also presented as well as an examination of their abilities to derepress certain carbon repressible enzymes.

Nitrogen repression of sporulation has been re-appraised in the wild-type in order to more clearly define the physiological parameters of repression. Attempts have been made, using the spd mutants which may be altered in nitrogen repression of sporulation,

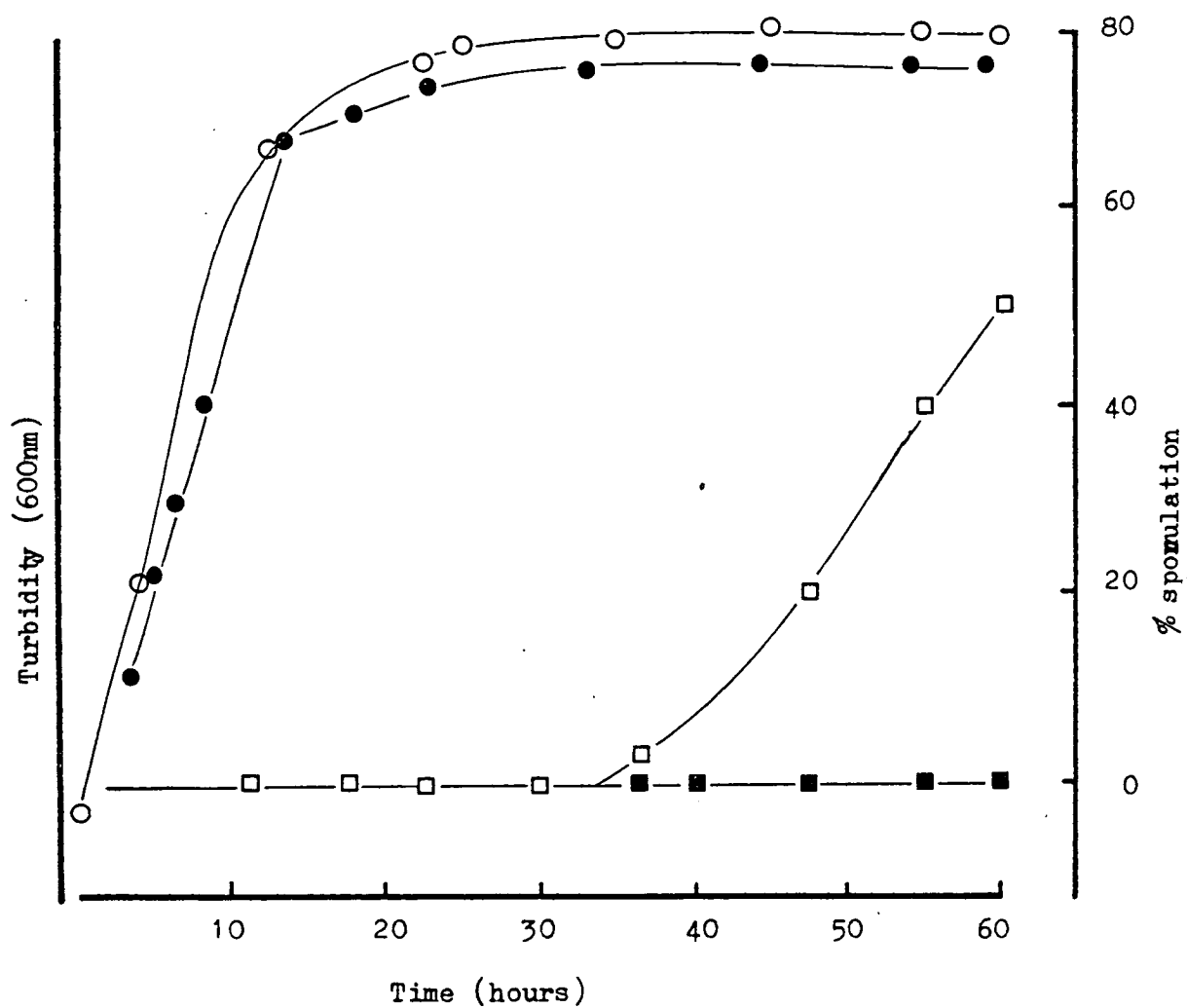


Figure 1.4

Growth and sporulation of wild-type and ID16D on YEPD.

( From Dawes 1975).

growth of S41; ○ growth of ID16D; ■ sporulation of S41;

□ sporulation of ID16D.

to establish a common mechanism of nitrogen control. Finally, revertants of the spd mutants have been studied genetically and biochemically with the aim of further elucidating the mechanism of control and its alterations.



## Chapter 2.

### MATERIALS AND METHODS.

2.1 Organism. The strains of Saccharomyces cerevisiae used in this study are presented in table 2.1. Definitions of genetical abbreviations are listed in table 2.2.

2.2 Culture conditions. A non-synthetic complete medium containing 2% bactopectone, 1% yeast extract and 2% glucose (YEPD) was used for maintenance of stocks and as a routine growth medium. When required the 2% glucose was substituted with 2% potassium acetate (YEPA), 2% glycerol vol/vol (YEPG) or 2% vol/vol ethanol (YEPE). In some experiments the YEP medium was employed at a five fold dilution (Dawes 1975).

For minimal culture a synthetic medium described by Wickerham (1946) was used. The basic mixture of trace elements, salts and vitamins is available commercially (Difco) and this was employed at 0.3%. A carbon source, 2%, nitrogen source, usually 7.5mM ammonium sulphate, and auxotrophic requirements,  $20\mu\text{g ml}^{-1}$  were added as described.

Sporulation medium comprised 1% potassium acetate with auxotrophic requirements at  $20\mu\text{g ml}^{-1}$ .

Solid medium was supplemented with 2% agar. All media were sterilised by autoclaving, 20 minutes at 15psi /  $120^{\circ}\text{C}$ . Glutamine and pyruvate were sterilised by membrane filtration, Millipore 0.45 $\mu$ m pore size, and added to sterile medium.

Cells were routinely grown at  $30^{\circ}\text{C}$ , liquid cultures were aerated by mechanical shaking.

Strains were maintained on YEPD slopes at  $4^{\circ}\text{C}$ ; cultures for

Table 2.1

## Description of strains.

Strain	Genotype	Origin	Remarks
S41	$\frac{a}{\alpha} \frac{D}{D} \frac{arg4-1}{arg4-1} \frac{cyh1}{cyh1}$	H.O. Halvorson Brandeis University	a homothallic diploid; parent of sporulation mutants.
DB1	$\frac{a}{\alpha} \frac{D}{D} \frac{his4}{his4}$	S41	a homothallic diploid; parent of sporulation mutant 7.4A.
ID16D	$\frac{a}{\alpha} \frac{D}{D} \frac{arg4-1}{arg4-1} \frac{cyh1}{cyh1} \frac{spd1-1}{spd1-1}$	isolated by U.V. mutagenesis from S41	characterised by extremely poor growth and hypersporulation on glycerol medium.
ID19D	$\frac{a}{\alpha} \frac{D}{D} \frac{arg4-1}{arg4-1} \frac{cyh1}{cyh1} \frac{spd1-2}{spd1-2}$	"	"
7.4A	$\frac{a}{\alpha} \frac{D}{D} \frac{his4}{his4} \frac{spd1-3}{spd1-3}$	isolated by irradiation from DB1	"
ts85	$\frac{a}{\alpha} \frac{D}{D} \frac{arg4-1}{arg4-1} \frac{cyh1}{cyh1} \frac{spd1-4}{spd1-4}$	isolated by U.V. mutagenesis from S41	"
SHID5C y209	$a ade6 ura1$ $\alpha ade5$	The Berkely Yeast Genetic Stock Culture Collection	Haploid tester strain
SMC-1A	$\alpha leu1 MAL2-8^C$	F.K. Zimmermann	Constitutive maltase producer; wild type of mutants altered in carbon catabolite repression.
CH.17-3c	$a leu1 MAL2-8^C MAL3$ $SUC3 cat1-1$	"	Constitutive producer of maltase carrying the cat1-1 mutation for an inability to derepress certain carbon catabolite repressible enzymes.
cat1.S3-14c	$\alpha his4 MAL2-8^C MAL3$ $SUC3 cat1-1$	"	"

Table 2.2

## Abbreviations.

a.	mating type a.
$\alpha$	mating type $\alpha$ .
ade.	adenine auxotroph.
arg.	arginine auxotroph.
cat.	genes involved in carbon catabolite repression; designated by F.K. Zimmermann.
cyh.	cycloheximide resistance.
d.	gene conferring homothallism; it is believed to cause, very soon after germination mutation of an adjacent cell, usually the bud, to the opposite mating type. Mating between the two then ensues. It generally results in formation of homozygous diploids in which recessive mutations can be detected.
his.	histidine auxotroph.
leu.	leucine auxotroph.
MAL.	maltose utilization.
MAL2-8 <sup>c</sup>	mutation conferring constitutive synthesis of maltase.
spd.	mutants which show derepressed sporulation.
spo.	mutants which do not sporulate or sporulate to an abnormally low frequency.
SUC.	sucrose utilization.
ura.	uracil auxotroph.

experiment were grown up from a loopful of cells from these storage slopes and mutant strains were tested for reversion in each experiment.

2.3 Growth measurement. Growth of cultures was measured by turbidity at 600nm, Pye Unicam SP600 spectrophotometer. Since the curve of cell numbers versus turbidity is non linear above 1.0 samples were diluted with water where necessary to a turbidity of 0.5-1.0.

2.4 Estimation of sporulation frequency. Percentage sporulation was measured by direct counting of asci and vegetative cells, x400 phase contrast, in several non-overlapping fields. A minimum of 400 cells were counted for each estimation no distinction being drawn between buds and mother cells.

2.5 Growth experiments. Log phase cells from overnight culture in YEPD were harvested by centrifugation; 2000xG, 3 minutes, washed in 0.1M potassium phosphate, pH 7.0, or with a five-fold dilution of YEP, and inoculated to a turbidity of 0.05-0.1 in the desired medium.

2.5 Carbon derepression experiments. Log phase cells from 1.0L of YEPD culture were harvested by centrifugation; 2000xG, 10 min, and resuspended to a turbidity of 6-7 in YEP-ethanol or YEP-glycerol. Samples from the culture were harvested as above and washed twice with 0.1M potassium phosphate, pH 6.5.

2.6 Nitrogen derepression experiments. The levels of nitrogen-repressible enzymes were determined on log-phase cells growing under three different regimes; minimal medium supplemented with

1) 2% glucose, 7.5mM ammonium sulphate

2) " " , 20mM amino acid

3) " , 20 mM amino acid.

10ml

Samples were harvested at a turbidity of 1.0 by filtration, Millipore 4.5 $\mu$ m pore size, and washed on the filter with three ten ml aliquots of distilled water prior to freeze drying.

The kinetics of derepression of nitrogen repressible enzymes were studied with cells grown in minimal medium with 2% glucose and 7.5mM ammonium sulphate as above. Cells were harvested by centrifugation; 1500xG, 5 minutes, washed with 0.1M potassium phosphate buffer and resuspended to the original turbidity, 1.0 unit, in glucose minimal medium with amino acid as sole nitrogen source.

## 2.7 Cell breakage.

a) Freeze drying of cells on filters was found to be an efficient method for the release of some enzymes, NAD<sup>+</sup> dependent glutamate dehydrogenase, arginase and glutamine synthetase. The technique was found to be most useful for serial samples taken at short time intervals which could be processed together with good reproducibility.

b) Sonication produced 30-50% cell breakage with four one minute bursts at maximum output ( MSE automatic ultrasonicator) with

~~The sonicate was centrifuged~~

### Note.

Sonication or Agitation with glass beads was carried out on 100ml samples washed and concentrated by centrifugation and resuspension in 0.1M potassium phosphate pH 6.5 to a volume of 5ml.

centrifugation and resuspension in 0.1M potassium phosphate, pH 6.5, were mixed with an equal volume of glass beads, BDH 40mesh,

in an 80ml glass centrifuge tube. Agitation with a Vibromixer, Shandon, model E1, fitted with a 2cm probe for four minutes while the sample was maintained at 4°C produced in excess of 80% cell breakage as judged by phase contrast microscopy. Glass beads were removed by centrifuging the lysate through stainless steel mesh and cell debris removed by centrifugation, 14,000xG 20 min. The supernatant was employed for the assay of glycerol kinase and glycerol 3-phosphate dehydrogenase.

2.8 Induction of sporulation. Cells grown with aeration at 30°C to mid log phase in YEPA or to early stationary phase in YEPD were harvested by centrifugation, resuspended in potassium acetate sporulation medium to a turbidity of 3-4 units in a flask of at least ten times the culture volume and shaken at 30°C, (Fast 1973).

## 2.9 Enzyme assays.

1) Arginase, E.C. 3.5.3.1. (L-arginine ureahydrolase), was assayed on freeze dried preparations essentially according to the method of Middlehoven (1964), except that the urea produced was estimated by the diacetyl monoxime method of Moore and Kauffman (1970).

Arginase requires metal ions, either  $\text{Co}^{2+}$  or  $\text{Mg}^{2+}$ , as cofactors and since the activation is a slow process this was accelerated by heating samples with 10mM magnesium chloride in 10mM Tris-HCL, pH 7.0, for 15 minutes at 50°C.

Samples were assayed in a final volume of 2.0ml containing 100µmoles of L-arginine hydrochloride, 200µmoles sodium glycinate, final pH 9.5 for 60 minutes at 30°C. The reaction was terminated by the addition of 2.5 ml of a mixture containing 300mls ortho-phosphoric acid, 100mls concentrated sulphuric acid, 0.237g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$

398 mls distilled water and 1.08 mls 0.1M  $\text{FeCl}_3$ .

The urea produced was estimated by the addition to the above acid/sample mixture of 0.25ml 3%w/vol diacetyl monoxime (2,3-butanedione monoxime). The tubes were mixed thoroughly and incubated in a boiling water bath for 30 minutes. Processed samples were allowed to come to room temperature in the dark and the formazan produced measured at 478nm. The colour was found to be stable for 24 hours in the absence of light.

Urea concentration was determined from a calibration curve run with each assay and enzyme activity expressed as  $\mu\text{moles urea produced min}^{-1} \text{ mg protein}^{-1}$ .

2)  $\text{NAD}^+$  dependent glutamate dehydrogenase, E.C. 1.4.1.2 (L-glutamate: NAD oxidoreductase [deaminating]), was assayed after the method of Ferguson and Sims (1971) by following the oxidation of NADH as  $A_{340\text{nm}}$  in a recording spectrophotometer (Unicam SP800).

Permeabilised cells were incubated for two minutes in 0.1M sodium phosphate buffer, pH 7.9, containing 0.5mM EDTA, with 24  $\mu\text{moles}$  of  $\alpha$ -keto glutarate and 25  $\mu\text{moles}$  of  $\text{NH}_4^+$  prior to the addition of 25  $\mu\text{moles}$  of NADH, final volume 1.6ml,  $30^\circ\text{C}$ .

Activity was expressed as  $\mu\text{moles NAD}^+_{\text{produced}} \text{ min}^{-1} \text{ mg protein}^{-1}$  using  $6.22 \times 10^3 \text{ Lmole}^{-1}$  as the extinction coefficient of  $\text{NAD}^+$ .

This assay was also carried out using cell free extracts obtained after sonication of samples in 0.1M sodium phosphate. The results obtained were similar to those obtained with permeabilised cells.

3) Glutamine synthetase, E.C. 6.3.1.2. (L-glutamate: ammonia ligase [ADP]), was assayed according to the method of Ferguson and Sims (1971).

Samples were incubated at 30°C in 2.0ml final volume of 0.05M Tris acetate with 0.5mM EDTA, pH6.4 containing 1.5µmole ADP, 70µmoles glutamine, 17.5 µmoles hydroxylamine, 4.5 µmoles manganous chloride and 35 µmoles sodium arsenate. The reaction was terminated by the addition of 1.0ml of a mixture of 10g trichloroacetic acid, 8.0g ferric chloride in 250ml 0.5M HCL.

Samples were centrifuged and absorbance measured at 500nm against a standard curve of amino acid hydroxamate formazan (L-glutamic acid  $\gamma$ -mono-hydroxamate) which was linear over the range used and activity expressed as  $\mu\text{moles formazan}_{\text{min}}^{-1} \text{mg protein}^{-1}$ .

4) NADP<sup>+</sup>-dependent glutamate dehydrogenase, E.C. 1.4.1.4 (L-glutamate: NADP oxidoreductase [deaminating]), was assayed by the method of Ferguson and Sims (1971) on cell free extracts after sonication.

Samples were assayed in 1.6ml sodium phosphate, pH 7.2, containing 25 µmoles ammonium chloride, 25 µmoles  $\alpha$ -keto glutarate and 0.25 µmoles NADPH; by following the oxidation of NADPH as change in  $A_{340\text{nm}}$  at 30°C. Activity was expressed as  $\mu\text{moles NADP}^+_{\text{min}}^{-1} \text{mg protein}^{-1}$ .

5) Isocitrate lyase, E.C. 4.1.3.1, was assayed by the method of Dixon and Kornberg (1959) with the modifications of Zimmerman et.al. (1977).

An appropriate volume of crude cell extract was added to 1.0ml of incubation buffer comprising 60mM potassium phosphate pH 6.5, 5mM magnesium chloride, 3.3mM phenyl hydrazine and 2mM cysteine. Change in  $A_{324\text{nm}}$  at 30°C was monitored and when the endogenous reaction had stopped 0.1ml of a solution of tri-sodium isocitrate,



12mg ml<sup>-1</sup>, was added . Activity was expressed as nmoles product min<sup>-1</sup> mg protein<sup>-1</sup> using  $1.7 \times 10^4$  Lmole<sup>-1</sup> for the molar extinction coefficient of the phenylhydrazone derivative of glyoxylate.

6) Malate dehydrogenase, E.C. 1.1.1.37 (L-malate: NAD oxidoreductase), was assayed by the method of Polakis and Bartley (1965).

Sample, 0.2ml cell extract, was incubated at 30°C with 1.0ml 60mM potassium phosphate pH 6.5, 0.2ml 10mg ml<sup>-1</sup> NADH until the endogenous reaction had stopped. The assay was initiated by the addition of 0.1ml of 3mg ml<sup>-1</sup> oxalic acid and followed by the change in A<sub>340nm</sub>. Activity was expressed as  $\mu\text{moles NAD}^+_{\text{produced}} \text{ min}^{-1} \text{ mg protein}^{-1}$ .

7) Glycerol kinase, E.C. 2.7.1.30, was estimated by the determination of <sup>14</sup>C glycerol 3-phosphate produced from <sup>14</sup>C glycerol after the method of Newsholme *et. al.* (1967) with the modifications of Sprague (1977).

Samples <sup>(10  $\mu$ l)</sup> were added to 0.1ml of incubation buffer containing 20mM magnesium chloride, 20mM ATP, 20mM sodium fluoride, 1.0mM EDTA, 20mM glycerol\*, 80mM Tris-HCL pH 8.0, and incubated for 15 minutes at 30°C. The reaction was terminated by the addition of two volumes of 95% ethanol and the samples placed on ice for 30 minutes.

Whatman DE81 filter paper (di-ethyl aminoethyl substituted) was cut into 2 cm squares to fit the Millipore filter tower and washed with 80mM Tris-HCL prior to application of the chilled ethanolic sample. The filters were subsequently washed with 10ml aliquots of 4%w/vol glycerol, three times with distilled water and once with 95% ethanol. The filters were dried prior to counting in PPO POPOP.

A blank containing incubation buffer was run for each assay and this value subtracted from sample readings and activity expressed

as nmoles glycerol 3-phosphate produced  $\text{min}^{-1} \text{ mg protein}^{-1}$ .

Glycerol\* was prepared by diluting  $^{14}\text{C}$  glycerol, Amersham U.K., 46mCi/mmol, with cold carrier glycerol to give 20mM final concentration at 1.0mCi/mmol.

8) Glycerol 3-phosphate dehydrogenase, E.C. 1.1.99.5, was assayed by the method of Lin et.al. (1962) which follows the rate of reduction of the tetrazolium dye MTT ([3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl] tetrazolium bromide) to its formazan as change in  $A_{550\text{nm}}$ . Phenazine methosulphate was employed as an electron carrier between the dehydrogenase and the dye and activity was further enhanced by adding potassium cyanide to block cytochrome dependent electron transport.

The reaction mixture contained 2.0ml 0.1M potassium phosphate 0.1ml  $\alpha$ -glycerophosphate (0.5M), pH 7.5, 0.1ml MTT (1mg/ml), 0.3ml phenazine methosulphate (1mg/ml), 0.2ml potassium cyanide (0.15M) and the reaction was initiated by the addition of sample to a total volume of 3.0ml. The assay was incubated at  $25^{\circ}\text{C}$  and activity expressed as  $\mu\text{moles MTT released min}^{-1} \text{ mg protein}^{-1}$  using  $8.1 \times 10^3 \text{ Lmole}^{-1}$  as the molar extinction coefficient for the formazan derivative of MTT.

#### 2.10 Protein determination.

Protein in extracts was estimated by the method of Lowry et.al. (1951).

0.2ml of sample was added to 1.0ml of copper tartrate reagent comprising 4% sodium carbonate (50ml), 2% sodium potassium tartrate (1.0ml), 1% copper sulphate (1.0ml) and 0.2M sodium hydroxide (50ml) and allowed to incubate at ambient temperature for 15 minutes. 0.1 ml of a 1:1 dilution of Folin-Ciocalteu's phenol reagent was subsequently added to each sample with

vigorous mixing and the colour allowed to develop for 90 minutes at ambient temperature. Protein concentration was established as  $A_{740\text{nm}}$  against a calibration curve.

Protein in whole cells was estimated by a modification of the above procedure in which the cell samples (0.1ml) are incubated with 1.0M sodium hydroxide containing 4% sodium deoxycholate (0.1ml), either overnight at 30°C or for one hour at 60°C. The sodium hydroxide in the copper tartrate reagent is replaced with distilled water in this case but the remainder of the procedure is as above.

## 2.11 Uptake and incorporation of exogenous substrates.

1) Acetate and glycerol uptake and incorporation during adaption to growth on 1/5 YEPA and 1/5 YEPG.

Strains were pregrown on YEPA, harvested in log phase, washed once and resuspended in the appropriate medium to a turbidity of 0.5.  $^{14}\text{C}$  labelled substrate was added to the growth medium to 0.4  $\mu\text{Ci ml}^{-1}$ . Samples (1.0ml) were taken at hourly intervals, centrifuged, washed with three 1.0ml aliquots of distilled water and two of 60% ethanol. A known volume (0.5ml) of each <sup>Supernatant</sup> ethanol<sub>A</sub> was used for the estimation of soluble pools and the ethanol<sub>A</sub> insoluble pellet redissolved in water (0.5ml) used for determination of incorporation into macromolecular components by scintillation counting in 5.0ml Instagel (Packard).

2) Total uptake studies.

Samples were filtered onto preautoclaved squares of filter (Millipore, 0.45 $\mu\text{m}$  pore size), washed with three 10ml aliquots of water, dried under an infra-red lamp and counted in a toluene based scintillator (4.0g 2,5-diphenyloxazole, 0.5g 1,4-Bis-[5-phenyloxazol-2-yl] benzene, to one litre of toluene).

## 2.12 Estimation of cyclic nucleotides.

Cyclic AMP ( Cyclic 3', 5'-adenosine monophosphate) levels were measured during adaption to glycerol and in the early stages of sporulation on cell free extracts prepared by the method of Sy and Richter (1972).

50 ml samples were centrifuged (0.1-0.2 g wet weight packed cells), washed once and resuspended in 2.0ml of 10% TCA. The samples were held on ice for 10 minutes, centrifuged and the pellet re-extracted with 1.0ml TCA. The supernatants were combined and mixed with 0.3ml HCL (1.0M) and 6.0ml ether. After 10 minutes on ice with intermittent mixing the ether phase was discarded and the aqueous phase re-extracted with 6.0ml ether, this step was repeated six times. Residual ether was blown off with a stream of nitrogen gas and a known volume of the aqueous fraction freeze dried.

The freeze dried aliquot from the above procedure was taken up in 1.0ml water and assayed for cyclic AMP by the competitive protein binding assay kit supplied by Boehringer (Mannheim).

## 2.13 Glycogen determination.

1) A rapid qualitative assay for glycogen based on iodine staining by the method of Chester (1967).

Cultures were plated on YEFD agar and incubated for three days at 30°C to permit the accumulation of glycogen by cells in late-log early stationary phase. The colonies were stained by flooding the plate with a 0.2% solution of iodine in 0.4% potassium iodide. Glycogen positive colonies appear redish-brown, the colour fading in approximately thirty minutes.



2) Carbohydrate fractionation and quantitative glycogen estimation was performed by the procedure of Kane and Roth (1974). Samples taken at intervals during incubation in sporulation medium were harvested by centrifugation, resuspended in 10mM HCL and stored at 5°C prior to analysis.

The cells were centrifuged and resuspended in 1M potassium hydroxide, heated for one hour on a boiling water bath and the alkalai soluble fraction separated from insoluble glycogen and glucan by centrifugation. The supernatant fraction containing trehalose, glycogen and mannan was further fractionated by the addition of two volumes of 98% ethanol which after overnight incubation at 5°C caused the precipitation of glycogen and mannan. This precipitate was collected by centrifugation, washed twice with 67% ethanol and taken up in water, and total carbohydrate determined using the Anthrone procedure below. Mannan was separated by the addition of an equal volume of Fehlings reagent\* and the precipitated mannan collected by centrifugation, washed and dissolved in a drop of 6M HCL followed by 0.5ml water. The carbohydrate in this mannan fraction was determined by the Anthrone procedure and glycogen estimated by the difference in the two determinations above.

3) Anthrone procedure, by a modification of the method of Mokrasch 1954.

1.0ml anthrone solution, 0.1g anthrone in 100ml 72% sulphuric acid, was added to 0.1ml test solution with 0.1ml distilled water. The mixture was cooled on ice then placed on a boiling water bath for 7 minute, cooled and the absorbance read at 625nm.

\*Fehlings reagent was made up immediately prior to use by mixing equal volumes of 7.3% copper sulphate and a solution of 34.6%

sodium potassium tartrate containing 25% potassium hydroxide.

#### 2.14 Genetic analysis.

The standard techniques of yeast genetic analysis were employed (Mortimer and Hawthorne 1969).

Crosses were carried out by mixing strains with different genetic markers on YEPD agar plates and selecting for diploids on minimal medium. Homothallic diploid strains were sporulated before mating. Diploids were sporulated on potassium acetate agar plates, a sample of asci treated with gluculase enzyme to digest ascospore walls and tetrads dissected on an agar slab.

Chapter 3.THE PHENOTYPES OF THE DERERESSED MUTANTS RELATIVE TO THAT OF THE WILD-TYPE.

In the introduction a mutant, isolated in this laboratory, was described as "sporulation-derepressed" spd due to its ability to sporulate extensively on YEP-glycerol medium whilst exhibiting little growth under these conditions. Sporulation also occurred after the end of growth on YEP-glucose. These features, not observed in the wild-type, were found to be characteristic for all the other spd1 mutants isolated. In order to establish the extent of the effects of the spd1 lesion a more complete study of the phenotype of the spd1 mutants relative to that of the wild-type is necessary. It is essential, for example, to determine if the poor-growth/derepressed-sporulation characters are present on carbon sources other than glycerol. Such analysis would elucidate the biochemical lesions present in the spd strains and also allow proposals to be made with respect to control mechanisms.

Growth and sporulation responses of spd mutants and wild type to different carbon sources.

After 2-3 days on YEP-glycerol agar the wild type, S41, showed very extensive growth and the mutant relatively little. A high frequency of asci was observed in the mutant after this time but not in the wild-type.

Another characteristic of all the spd mutants is their capacity to sporulate heavily on rich glucose medium after stationary phase is reached (Figure 1.4). After 72 hours incubation in YEPD approximately 50-60% spores were present in spd mutants ID16D,

ID19D and 7.4A but none in the wild-type S41. Prolonged incubation over 4-5 days, however, led to the appearance of a low percentage of asci in the wild-type, although most of the cells became vacuolated and gave the characteristic appearance of starved cells. This can be taken to indicate that S41 became limited for some component of the medium but remained repressed for sporulation by some other factor(s).

As sporulation in the spd mutants only occurred after growth on glucose but during growth on glycerol it may be that the poor-growth/ derepressed-sporulation characters are only observed in combination, on carbon sources whose utilisation is subject to carbon catabolic repression as discussed in the Introduction (Chapter 1). Various other carbon sources were tested for their effect on the expression of the spd phenotype. As the strains used here, including the wild-type, do not grow on minimal medium with acetate or glycerol as carbon source a five-fold dilution of YEP medium (Dawes 1975) containing the appropriate carbon source was used. Full strength YEP medium permitted growth of the spd mutants to a variable extent and, as YEP is a very rich medium, diluting it shortened considerably the time course of the experiment. Turbidity measurements were also more accurate as the need for dilution was minimised. Table 3.1 lists the growth and sporulation responses observed using different carbon sources at various times after resuspension of log phase glucose-grown cells to a low turbidity, (0.05 units).

spd mutant ID16D was observed to grow very poorly on acetate, glycerol, lactate, pyruvate and succinate but nearly as well as the wild-type S41 on ethanol, fructose, galactose and glucose.



Carbon source	24 hours				28 hours				48 hours			
	Turbidity		% Sporulation		Turbidity		%Sporulation		Turbidity		% Sporulation	
	S41	ID16D	S41	ID16D	S41	ID16D	S41	ID16D	S41	ID16D	S41	ID16D
Acetate	0.56	0.28	8	39	0.59	0.28	15	75	0.61	0.29	55	90
Ethanol	1.2	1.1	0	0	--	--	--	--	6.7	6.0	1	5
Fructose	5.5	5.0	0	0	--	--	--	--	7.4	6.6	0	17
Galactose	1.5	0.8	0	0	--	--	--	--	7.8	6.0	0	2.3
Glucose	4.88	4.72	0	0	--	--	--	--	8.3	7.2	1	10
Glycerol	0.77	0.30	0	2	--	--	--	10	0.74	0.29	20	88
Lactate	0.32	0.15	0	0	--	--	--	--	0.35	0.135	8	11
Pyruvate	0.89	0.46	0	1	0.9	0.455	1	11	0.86	0.395	11	88
Succinate	0.15	0.075	5	30	0.135	0.095	5	36	0.12	0.095	47	75

Table 3.1 Growth and sporulation of S41 and ID16D in various carbon sources with YEP as nitrogen source.

S41 and ID16D were harvested during log phase growth on YEPD (turbidity 8.0) and washed twice with a five-fold dilution of YEP. The cells were resuspended in 1/5 YEP + 1% carbon source to a turbidity of 0.05-0.1 and samples taken at the indicated times.

S41 also grew poorly on lactate and succinate in comparison to other non-fermentable carbon sources but even this limited growth was significantly better than that of ID16D. It was also obvious that all the non-fermentable carbon sources, with the exception of ethanol, supported less growth than did fermentable carbon sources at similar concentrations. Therefore it is probable that the nitrogen source was still present in excess with those carbon sources which permit relatively little growth of both wild-type and mutant. Other spd mutants, ID19D and 7.4A, have also been shown to grow to the same extent as S41 and ID16D on YEP-ethanol.

On those carbon sources on which the spd1 mutant ID16D grew poorly compared to the wild-type asci were present by 28 hours after resuspension and increased to a high frequency by 48 hours. Acetate promoted very good sporulation in the mutant, 39% spores were present at 24 hours rising 75% at 28 hours and 90% at 48 hours. By comparison only 8% asci were observed in the wild-type culture at 24 hours and 15% at 28 hours although this rose to 55% by 48 hours. spd mutant ID16D also sporulated extensively on succinate, pyruvate and glycerol while the wild-type S41 did not even by 48 hours. Lactate proved to be unable to support good sporulation of either S41 or ID16D although a growth difference was apparent between the two strains.

In those carbon sources on which the spd1 mutant grew, glucose, galactose, fructose and ethanol, sporulation was not observed until 48 hours after resuspension. The wild-type did not exhibit sporulation under these conditions. This indicates that initiation of sporulation in the spd1 mutant ID16D did not take place until after the end of growth on these carbon sources.

Similarly abnormal sporulation, in sporulation medium, takes approximately 24 hours to completion (Figure 3.1), initiation of sporulation in the media on which the mutant grew poorly must have taken place very soon after the transfer in order for asci to be present by 28 hours. As log phase glucose grown cells do not sporulate on transfer to sporulation medium (Fast 1973) the extra time involved probably reflects a necessary period of adaptation, possible in YEP medium but not in sporulation medium. Supporting this proposal is the observation that logarithmically growing cells of spd1 mutant ID16D in YEPD did not sporulate on transfer to sporulation medium.

The growth and sporulation kinetics of wild-type S41 and spd1 mutants ID16D and ID19D on YEP medium containing pyruvate and glycerol as carbon sources are illustrated in figures 3.2 and 3.3.

Again growth was found to be reduced in both the spd1 mutants as compared to the wild-type. Both spd1 mutants were, however, similar in their growth and sporulation profiles, although ID16D appeared to sporulate to a greater extent than did ID19D. Sporulation in S41 occurred at a later time and to a reduced frequency. The rate of appearance of asci in the spd1 mutants <sup>was similar</sup> to that observed for both ID16D and S41 in sporulation medium, (Figure 3.1). Clearly asci were formed at the same rate in the mutant and wild-type, however, ID16D began to form tetrads at an earlier time. This might either reflect a difference in the rate at which the mutant and wild-type adapt to the medium shift or a preadaptation of ID16D cells for sporulation during late-log/early-stationary growth in YEPD medium, from which transfer to sporulation medium was carried out.

A difference in appearance between S41 and ID16D was often

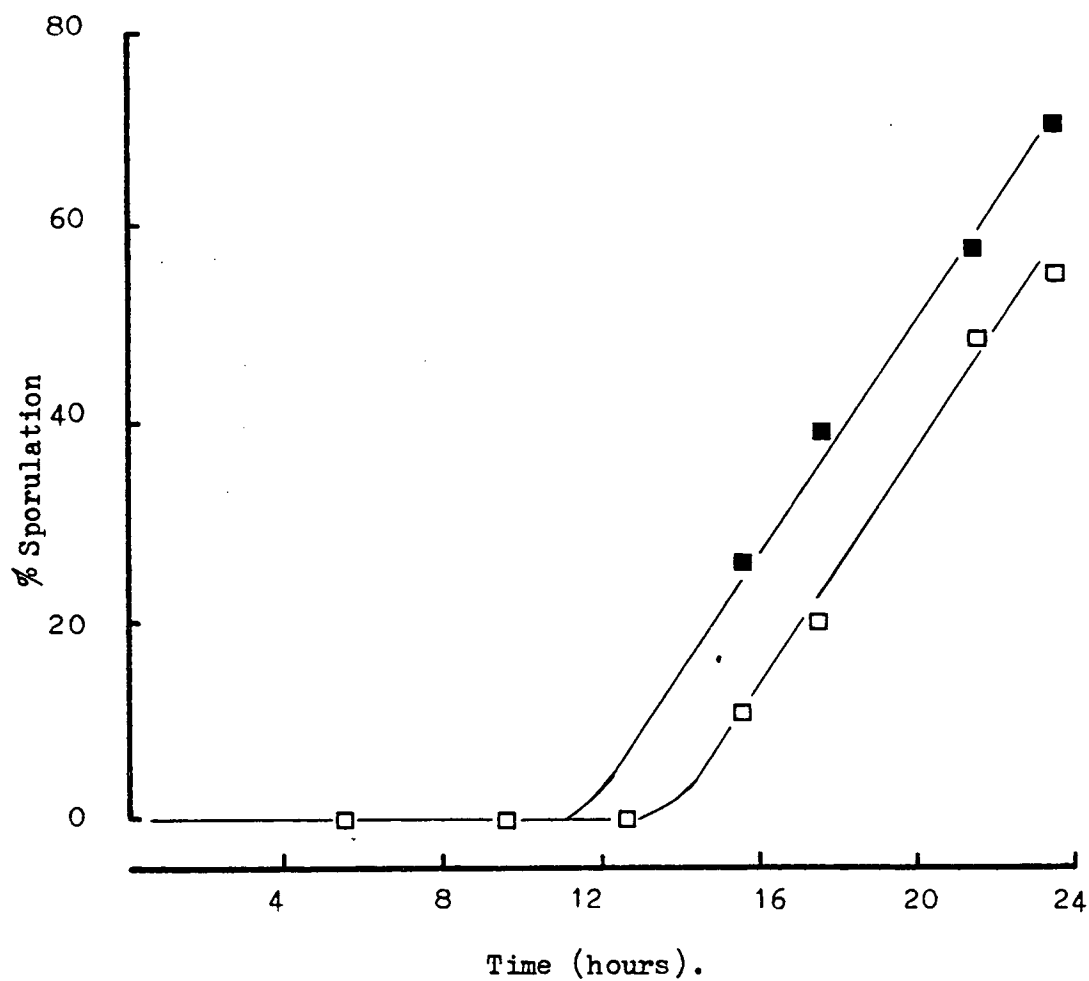


Figure 3.1

Kinetics of appearance of asci in 1% potassium acetate sporulation medium after transfer of early-stationary YEPD grown cells. □ S41; ■ ID16D.

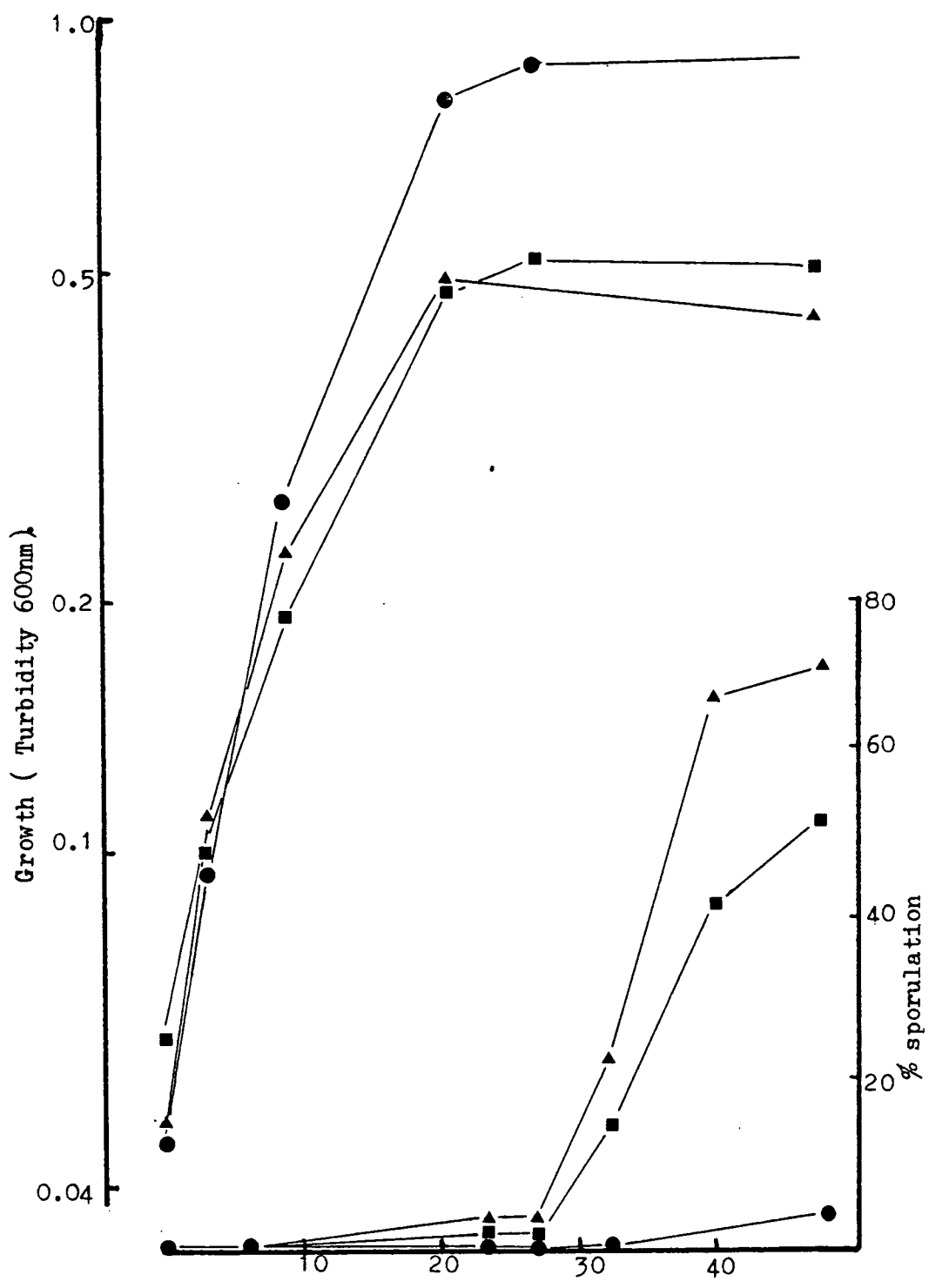


Figure 3.2

Growth and sporulation of ● wild-type S41, and derepressed strains ▲ ID16D and ■ ID19D on pyruvate containing medium. Cultures pre-grown in YEPD were resuspended to a similar low turbidity in a five-fold dilution of YEP-pyruvate medium.

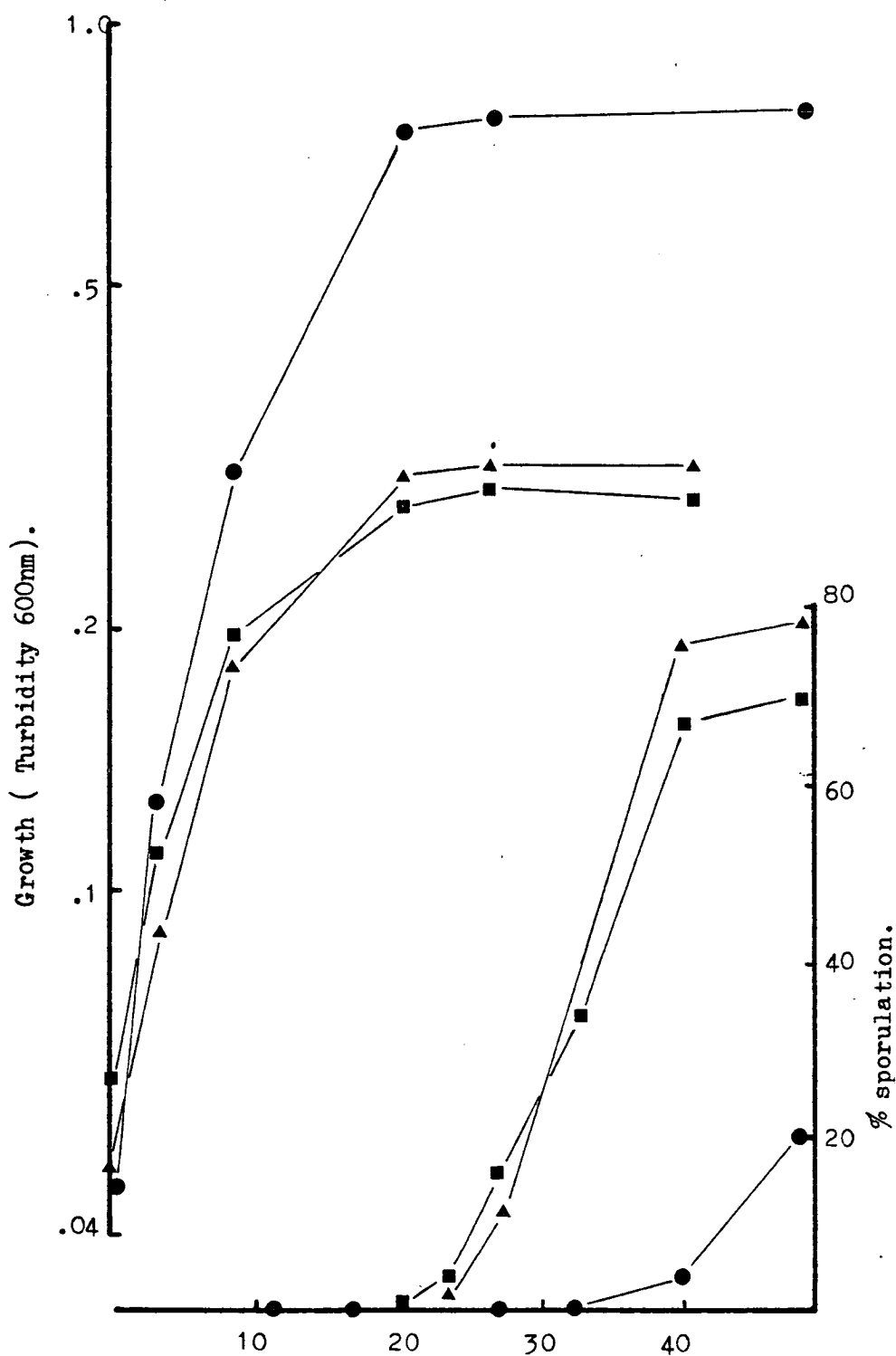


Figure 3.3

Growth and sporulation of ● wild-type S41 and derepressed strains ▲ ID16D and ■ ID19D on glycerol containing medium. Cultures were pre-grown in YEPD and resuspended to a similar low turbidity in a five-fold dilution of YEP-glycerol medium.

noted during growth and sporulation on the different carbon sources. On glycerol for example although S41 sporulated to 20% by 48 hours (where ID16D was 90%), the sporulated cells consisted of mainly two-spored asci whereas ID16D showed the normal four-spored configuration. The significance of this is not clear but as the four-spored ascus is indicative of normal sporulation formation of the two-spored may reflect a less efficient form of sporulation. Ethanol grown wild-type S41 showed no sporulation but 60% of the cells were noted to contain phase-dark granules and to be elongated in appearance. ID16D on the other hand had produced 8% of normal spores by 48 hours while the remainder of the population was composed of large, round non-vacuolated cells. These large, round non-vacuolated cells are typical of cultures which are undergoing sporulation. These features were observed also with galactose as carbon source.

In an attempt to obtain an earlier, more synchronous form of sporulation by the spd1 mutant in the carbon sources on which it grew poorly strains were inoculated into the same media to a much higher initial turbidity, 0.5-0.6 units, (Table 3.2). The results were, however, almost identical to those obtained with the low inoculum. This supports the previous proposal that a certain preparation time is required for sporulation under these conditions.

Growth and sporulation responses of wild-type and derepressed strains to YEP in the absence of a carbon source.

When spd1 mutant ID16D and wild-type S41 were resuspended in a five-fold dilution of YEP in the absence of a carbon source only a small increase in turbidity was observed over a 24 hour period (Table 3.2).

Carbon source	24 hours		48 hours		90 hours	
	Turbidity	% Sporulation	Turbidity	% Sporulation	Turbidity	% Sporulation
	S41 16D	S41 16D	S41 16D	S41 16D	S41 16D	S41 16D
Acetate	.93 .69	0 7	.93 .62	5 60	.92 .62	6 61
Ethanol	1.35 1.3	00 0	6.5 6.2	0 1	6.4 5.9	0 16
Fructose	--- ---	0 0	8.4 7.4	0 0	8.4 7.9	0 2
Galactose	7.6 3.9	0 0	10.0 9.0	0 0	8.9 7.8	0 1
Glucose	--- ---	0 0	8.1 8.0	1 1-2	8.1 7.8	0 1
Glycerol	.80 .56	1 6	.74 .57	29 24	.66 ---	34 50
Lactate	2.0 .88	0 0	7.4 3.6	0 0	6.9 3.4	0 0
Pyruvate	.96 .69	0 14	.78 .59	6 58	.66 .58	6 70
Succinate	.68 .51	0 10	.70 .48	0 22	--- .43	1 21
YEP	.76 .64	0 10	.75 .58	0 16	.74 .56	0 19

Table 3.2 Growth and sporulation of S41 and ID16D on a five-fold dilution of YEP + carbon source.

Strains were pregrown in YEFD, harvested, washed and re-inoculated in the appropriate medium to a turbidity of 0.59 for S41 and 0.55 for ID16D.



Sporulation was completely absent in S41, the cells containing a single phase-dark inclusion. ID16D formed 20% two or three-spored asci. Thus it appears that even in the presence of YEP ID16D has the capacity to sporulate but that S41 does not. The low frequency of asci in the former case does, however, emphasise the need for an exogenous carbon source for optimum spore formation.

Sporulation using carbon sources other than acetate in the sporulation medium.

Sporulation is usually induced by transferring cells to a sporulation medium containing 1% potassium acetate. Some methods include a low concentration of of Yeast Extract but this was found to be unnecessary for sporulation in our strains. Under these conditions pregrowth in a medium conducive to subsequent sporulation (such as YEP-acetate) is required to pre-adapt cells to non-fermentative metabolism (Fast 1973). However, it has also been demonstrated that sporulation is possible following transfer from an early-stationary phase culture grown on YEP-glucose (YEPD) medium (Fast 1973). In view of the response of the spd1 mutants to certain carbon sources this latter procedure was employed when spd1 and wild-type strains were transferred to sporulation medium. In this way it was hoped to minimise any effect of the spd1 on the potassium acetate sporulation profile. This procedure may not be ideal, however, as the slightly earlier appearance of asci in ID16D in sporulation medium (Figure 3.1) following transfer of early-stationary phase cells from YEPD may indicate either that some of the cells had begun to initiate sporulation or that some form of preadaptation had taken place prior to transfer.

Although sporulation is usually studied using acetate as carbon source there appears to be no reason why other non-fermentable carbon sources could not serve equally well. Indeed it is necessary, in view of the previous results on growth and sporulation, to establish which carbon sources can promote sporulation and which, if any, can repress it. For example Miller and Halpern (1956) observed that the addition of ethanol to potassium acetate sporulation medium was inhibitory to sporulation at concentrations comparable to those for glucose inhibition. Alternatively Arnaud *et.al.* (1972) maintain that ethanol is as readily utilised for sporulation as acetate, since ethanol is metabolised by way of acetate. It may be that those carbon sources whose utilisation is subject to carbon catabolite repression, on which the spd mutant grows, may also be those that can inhibit sporulation.

As several of the carbon sources used here were non-ionic a weak phosphate buffer was included in the sporulation medium, (0.067M potassium phosphate, pH 7.0: Vezhinet *et.al.* 1974). Carbon sources replacing acetate were present at 1%.

It is obvious from Table 3.3 that acetate promoted the most efficient sporulation of both S41 and ID16D, 42% and 50% respectively at 24 hours. None of the other carbon sources investigated gave spore formation except ethanol with ID16D, 15% at 24 hours. Most of the carbon sources other than glucose and fructose permitted a limited amount of sporulation to occur by 40 hours, although in no case was this comparable to that obtained with acetate. On a few carbon sources ID16D sporulated slightly better than did S41 (ethanol, lactate and succinate) but asci formed on succinate while recognisable were very abnormal in appearance indicating

	% Sporulation			
	24 hours		40 hours	
	S41	ID16D	S41	ID16D
Carbon source				
Acetate	42	50	60	68
Ethanol	0	15	18	40
Fructose	0	0	0	1-2
Galactose	0	0	27	20
Glucose	0	0	0	2-3
Glycerol	0	0	20	10
Lactate	0	0	6	15
Pyruvate	0	0	12	10
Succinate	0	0	5	50
Phosphate	0	0	0	0

Table 3.3 Sporulation of S41 and ID16D employing different carbon sources in the sporulation medium. The medium contained 0.006M potassium phosphate, pH 7.0, arginine at auxotrophic requirement concentration and 1% carbon source. Early-stationary phase cells were harvested by centrifugation, washed once in the phosphate buffer and resuspended in the appropriate medium to a turbidity of 0.8.

that sporulation had not occurred with any degree of efficiency.

Therefore it would seem that acetate supports optimal sporulation in comparison with these other carbon sources. With the exception of ethanol and succinate the sporulation frequencies obtained with S41 and ID16D are similar for each carbon source. This suggests that the regulation of sporulation under each set of conditions may be similar in both mutant and wild-type. Certainly the ability of the spd1 mutant to sporulate on pyruvate and glycerol alone is very low compared to its ability to sporulate on these carbon sources in combination with YEP, (Table 3.1).

Growth rates of wild-type and derepressed strains on defined media.

The previous experiments on complex media provided a rapid test for growth and sporulation capacity but did not indicate possible differences in growth rates on carbon sources which supported good growth of both mutant and wild-type.

The growth rates of both mutant and wild-type were, however, identical on glucose minimal medium (Figure 3.4) although as on YEP-glucose (Figure 1.4) ID16D attains a higher turbidity than does S41. This may not be significant in that it could reflect a difference in cell shape. Similarly on galactose minimal medium (Figure 3.4) the growth rates of mutant and wild-type were comparable.

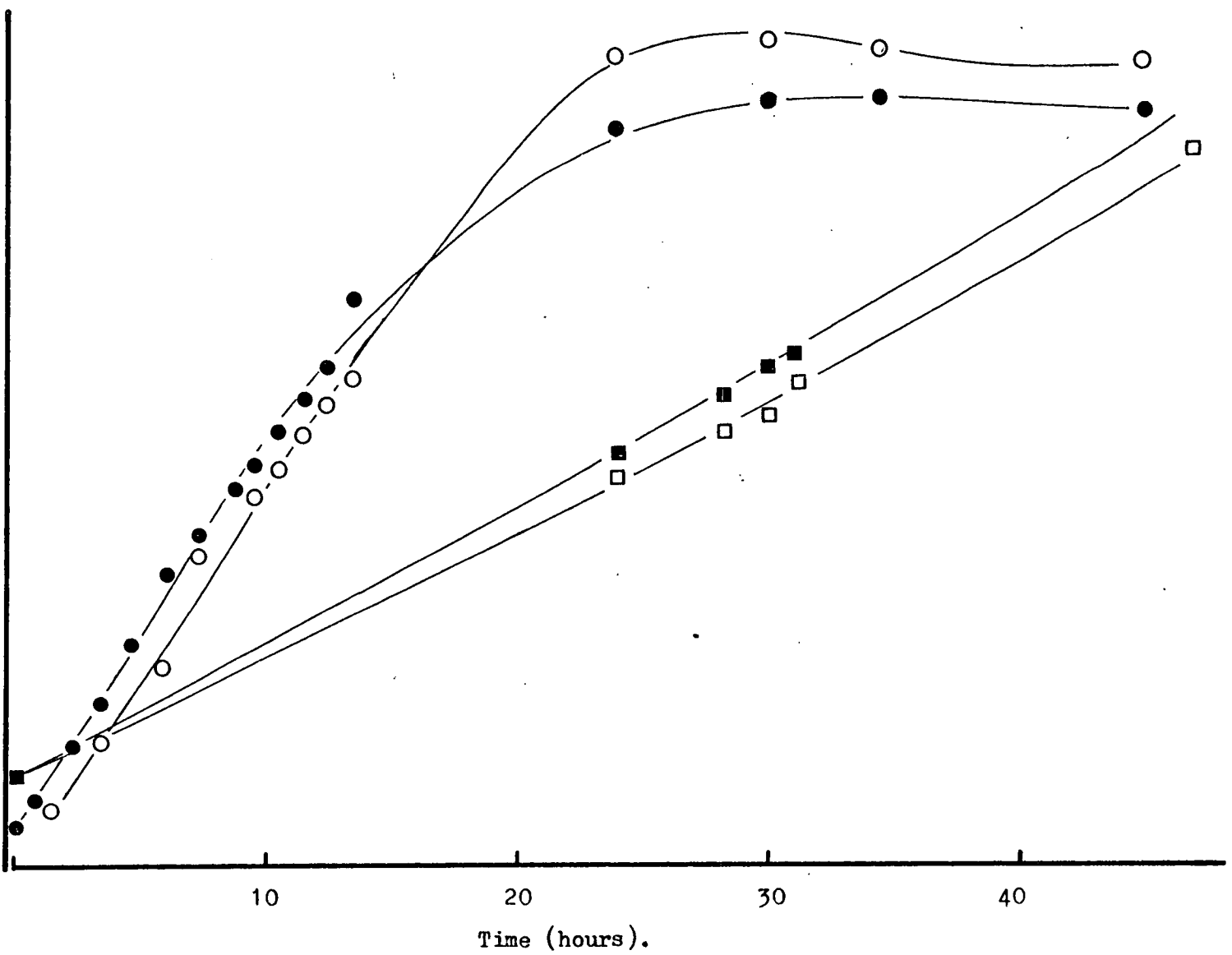


Figure 3.4

Growth rates of S41 and ID16D on glucose and galactose minimal media. ● S41 glucose; ○ ID16D glucose; ■ S41 galactose; □ ID16D galactose.

### Discussion.

One important phenotypic character of the spd1 mutation is the inability of homozygous spd1 diploids to grow normally on most non-fermentable carbon sources. With galactose, whose utilisation is subject to carbon catabolite repression although fermentable, ethanol, glucose and fructose there is a tendency for sporulation to occur in the mutant during stationary phase which is not observed in the wild-type.

As the same concentration of YEP as nitrogen source can support growth of the wild type to a high turbidity on glucose for example but not on acetate, glycerol etc. it seems likely that nitrogen sources in the form of the amino acids from the YEP remain in excess after cessation of growth on these poorer carbon sources. The mutant has a high tendency to sporulate rather than grow under these conditions which is absent in the wild-type. The lesion may therefore represent a gene which is involved in the nitrogen control of sporulation thus suggesting the reduction in sensitivity or abolition of this control in the mutant. Despite the obvious need for a carbon source for successful sporulation the ability of the spd1 mutant to achieve some degree of sporulation on YEP alone, when the wild-type does not, supports this hypothesis. Indeed it seems essential for YEP to exert some form of repression on sporulation as the wild-type can grow normally on YEP-acetate, but sporulate normally on acetate alone. Also promoting this theory is the observation of Dawes (1975), mentioned in the introduction (see Chapter 1), concerning sporulation in glucose minimal media with differing concentrations of  $\text{NH}_4^+$  and glucose.

Alternatively the poor growth of the spd1 mutants on carbon

sources subject to carbon catabolite repression of their utilisation might suggest that the spd1 mutation causes a pleiotropic inability to derepress the enzymes concerned with their metabolism i.e. carbon hyper-repressed. Such mutations have been described in yeast by Zimmermann (1977) and in *Aspergillus* where nitrogen repressible enzymes are pleiotropically affected (Arst and Cove 1973, see also Chapter 1). These control mutations are also recessive and result in defective growth on some but not all carbon and nitrogen sources. Therefore the ability of the spd1 mutants to grow in galactose and ethanol could support this hypothesis.

However, as a carbon source is essential for sporulation and the mutants do either grow or sporulate on all carbon sources it doubtful that they are altered in their abilities to metabolise them. The theory is further complicated by the observations that pyruvate and glycerol by themselves do not support good sporulation in either mutant or wild-type but do in the mutant in combination with YEP.

These considerations are further investigated in subsequent chapters.

#### Chapter 4.

### NITROGEN REPRESSION OF INDUCIBLE ENZYME SYNTHESIS IN WILD-TYPE AND SPORULATION-DEREPRESSED STRAINS.

Sporulation is sensitive to the presence of nitrogen compounds (Miller 1963) and as described in the Introduction (Chapter 1) there are many documented examples of enzymes whose synthesis is repressible by nitrogen sources. The two processes appear to be very similar in their response to ammonium ion; sporulation can be completely suppressed by the presence of relatively low concentrations of ammonium ion (see Chapter 5) and the levels of many nitrogen catabolising enzymes are extremely low in the presence of ammonium ion. Sporulation is likewise inhibited by rich amino acid mixtures as present in YEP while certain enzymes, arginase, urea amidolyase, allantoinase, are subject to repression by readily utilisable amino acids such as asparagine and glutamine in addition to ammonium ion (Dubois et.al. 1977).

It is not yet known if there exist any control elements which are common to both the nitrogen control of sporulation and to the nitrogen control of inducible enzyme synthesis. In this chapter this possibility is examined using the spd1 mutation. This, if it does as seems possible from the results of the previous chapter, represent a control lesion which reduces the sensitivity of sporulation to nitrogen, may also lead to a complete or partial release of nitrogen repression of enzyme synthesis in the presence of a repressor. Such an effect, if established, would confirm that nitrogen repression of sporulation is altered by the lesion and would also contribute to a greater understanding of the



mechanism of general nitrogen regulation in eukaryotic systems.

Enzyme levels in sporulation derepressed strains under different repression conditions.

Three enzymes, arginase,  $\text{NAD}^+$ -dependent glutamate dehydrogenase and glutamine synthetase, were selected for study since they are all subject to repression by ammonium ion (Middelhoven 1968, 1970; Ferguson and Sims 1971; Prival *et.al.* 1973) yet serve diverse roles in nitrogen metabolism. Arginase is not central to nitrogen metabolism while the other two enzymes are. Dubois *et.al.* (1973) suggested that arginase synthesis might be regulated in a different way to that of  $\text{NAD}^+$ -dependent glutamate dehydrogenase since although both are subject to repression by ammonium, a mutation in the  $\text{NADP}^+$ -dependent glutamate dehydrogenase leads to the derepression of arginase but not of  $\text{NAD}^+$ -dependent glutamate dehydrogenase in the presence of ammonium ion. Subsequently this group (Dubois *et.al.* 1977) suggested the existence of three types of nitrogen catabolising enzymes. Thus the *spd1* mutants could reflect lesions in the regulation of one group of enzymes but not others. In addition arginine and glutamate are good nitrogen sources in this organism and are thus experimentally convenient.

Strains were grown under three sets of conditions:

1. 2% glucose, 7.5mM ammonium sulphate.
2. 2% glucose, 7.5mM ammonium sulphate, 20mM amino acid.
3. 2% glucose, 20mM amino acid.

In the first medium only basal levels of these enzymes should be present while the second should reflect the sensitivity of the repression/induction to ammonium ion. The last regime, where the substrate serves as sole nitrogen source should reflect the fully induced level.

Threonine dehydratase has also been reported to be subject to repression by ammonium in some yeast strains (Holzer et.al. 1964) but only a small effect of ammonium ion was observed (Table 4.1) when this enzyme was assayed under the above conditions in our strains. This absence of repression of threonine dehydratase by ammonium has also been described for other strains of S. cerevisiae (Dubois et.al. 1973).

In the wild-type S41 arginase was induced 5-6 fold by growth on arginine as sole nitrogen source (Table 4.2) and 7.5mM ammonium sulphate repressed to approximately half this induced level.

Growth on glutamate as sole nitrogen source led to a seven-fold induction of  $\text{NAD}^+$ -dependent glutamate dehydrogenase (Table 4.3) and a four-fold increase in glutamine synthetase (Table 4.4). Both enzymes were, however, more subject to repression by ammonium than arginase.

In the mutant strains (Tables 4.2-4) the ammonium repression appears to be similar to that for the wild-type, thus there appears to be no effect of the spd lesion on the sensitivity of the strains to repression of enzyme synthesis by ammonium under these conditions. Activities obtained with some of the spd mutants were lower than those of the wild-type, intermediate levels of arginase in ID16D and ID19D (Table 4.2) and of glutamine synthetase in ID19D growing on glutamate as sole nitrogen source (Table 4.4). Although these lowered values were observed consistently in repeated experiments it is rather difficult to establish if these rather small differences in magnitude are a significant feature of the spd mutation.

It should be noted at this point that different experiments

Table 4.1

Effect of growth conditions on threonine dehydratase levels in wild-type S41 and sporulation derepressed strains ID19D and ID16D.

Growth conditions	S41	19D	16D
Glucose + $\text{NH}_4^+$	12.2	8.2	5.8
Glucose + $\text{NH}_4^+$ + threonine	16.6	10.0	8.3
Glucose + threonine	7.6	9.8	7.0

Growth conditions are described in section 2.6, specific activity was expressed as  $\mu\text{moles product} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$

Note.

Threonine dehydratase was assayed according to Greenberg D.M.(1962) in "Methods in Enzymology Vol V" S.P.Colowick and N.O.Kaplan eds., Academic Press, New York p 946-956.

Growth conditions	S41	7.4A	ID16D	ID19D
Glucose + NH <sub>4</sub> <sup>+</sup>	0.18	0.16	0.15	0.1
Glucose + NH <sub>4</sub> <sup>+</sup> + arginine	0.65	0.51	0.36	0.39
Glucose + arginine	0.96	0.99	0.89	0.72

Table 4.2 Induction and repression profiles of arginase in wild-type and sporulation derepressed strains.

Cultures were grown and harvested as described in section 2.6. Arginase was assayed as detailed in 2.9.1 and specific activity expressed as  $\mu\text{moles urea produced. min}^{-1} \cdot \text{mg protein}^{-1}$ .

Growth conditions	S41	7.4A	ID16D	ID19D
Glucose + $\text{NH}_4^+$	3.86	5.76	6.72	4.82
Glucose + $\text{NH}_4^+$ + glutamate	5.76	7.71	8.29	5.98
Glucose + glutamate	28.94	36.65	32.79	23.15

Table 4.3

Induction and repression profiles of  $\text{NAD}^+$ -dependent glutamate dehydrogenase in wild-type and sporulation derepressed strains.

Cultures were grown and harvested as described in section 2.6. Samples were assayed as detailed in 2.9.2 and specific activity expressed as  $\text{nmoles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$

Table 4.4

Induction and repression profile of glutamine synthetase  
in wild-type and sporulation derepressed strains.

Growth conditions	S41	ID16D	ID19D
Glucose + $\text{NH}_4^+$	0.186	0.14	0.17
Glucose + $\text{NH}_4^+$ + glutamate	0.203	0.09	0.22
Glucose + glutamate	0.88	0.79	0.66

Cultures were grown and harvested as described in section  
2.6. Samples were assayed as detailed in 2.9.3 and specific  
activity expressed as  $\mu\text{moles product} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$

were subject to variation in overall activities, particularly in the case of arginase, but that the same proportionality was observed between the three growth conditions in each experiment. This variability is probably due to inconsistency in the freeze-drying / permeabilisation régime necessitated by the large numbers of samples processed. In order to correct for this factor a wild-type control was processed in parallel with the mutants in each experiment. The assay system for arginase may also be a contributory factor as Middelhoven (1970) using a similar method also observed such differences in activity between experiments.

#### Kinetics of derepression.

Although the final enzyme levels attained by the mutants and wild-type under the three growth régimes above were similar, this method of analysis would not reflect differences in the rates of enzyme derepression. spd mutants could induce faster than wild-type as has been described for mutants altered in the regulation of carbon catabolite repression (Zimmermann *et.al.* 1977). These mutants carrying the CAT1-2<sup>d</sup> or cat2-1 lesions (see Chapter 1) exhibited faster derepression kinetics of carbon repressible enzymes when switched to a medium containing a non-repressing carbon source although they were still subject to normal repression in the presence of glucose.

The derepression kinetic profiles of the spd1 mutants were obtained following the transfer of cells from glucose-ammonium sulphate minimal medium to medium containing the inducing amino acids as sole nitrogen source. S41 and ID16D showed similar rates of induction for  $\text{NAD}^+$ -dependent glutamate dehydrogenase (Figure 4.1)

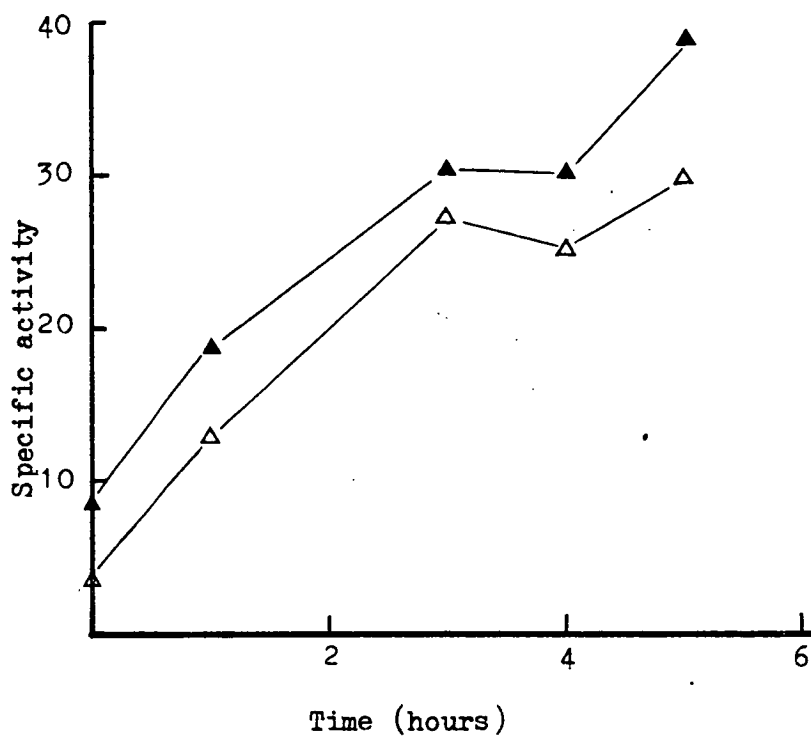


Figure 4.1

Induction kinetics of NAD<sup>+</sup>-dependent glutamate dehydrogenase. Specific activity is expressed as nanomoles.min<sup>-1</sup>.mg protein<sup>-1</sup>

▲ S41;    △ ID16D.



and glutamine synthetase (Figure 4.2) although once again the slight reduction in levels in ID16D was apparent. Repression rates of fully induced levels of glutamine synthetase by 7.5mM ammonium sulphate were also shown to be similar (Figure 4.3). Thus under these conditions the rate of production of these enzymes is unaltered by the spd1 mutation.

#### Carbon source effects on ammonium ion repression.

The experiments above were carried out on media containing glucose and sole carbon source and it is possible that the spd phenotype cannot be expressed in the presence of glucose. Thus the lesion may only be fully expressed or "switched on" when the glucose in the medium is used up or the carbon source is non-repressing. The high sporulation of spd mutants after growth on YEPD and their inability to grow on glycerol could be used as evidence for these proposals. However, if the spd mutation also had an effect on the regulation of nitrogen repressible enzyme synthesis there seems to be no reason why it should not show its influence on the synthesis in the presence of glucose. For arginase at least there appears to be no catabolite repression effect on synthesis as levels were found to be similar in galactose, lactate and ethanol with ammonium ion (Middelhoven 1970), thus indicating that arginase levels in S.cerevisiae are subject only to nitrogen repression. If, however, there is an effect of the spd1 lesion on nitrogen repressible enzyme synthesis with carbon sources other than glucose it would indicate that the mutation is not expressed at all on glucose and that there is a glucose effect on such synthesis which is epistatic to the spd gene.

Testing this proposal posed some difficulties since those

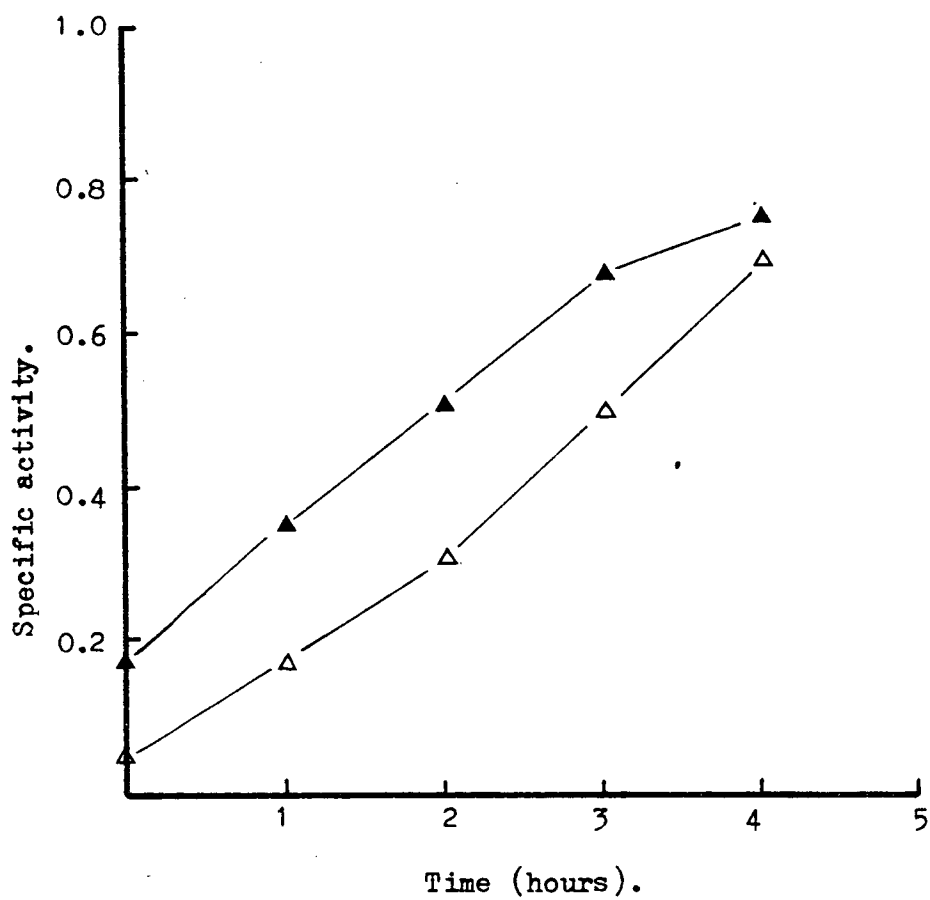


Figure 4.2

Induction kinetics of glutamine synthetase. Specific activity is expressed as  $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ .

▲ S41; △ ID16D.

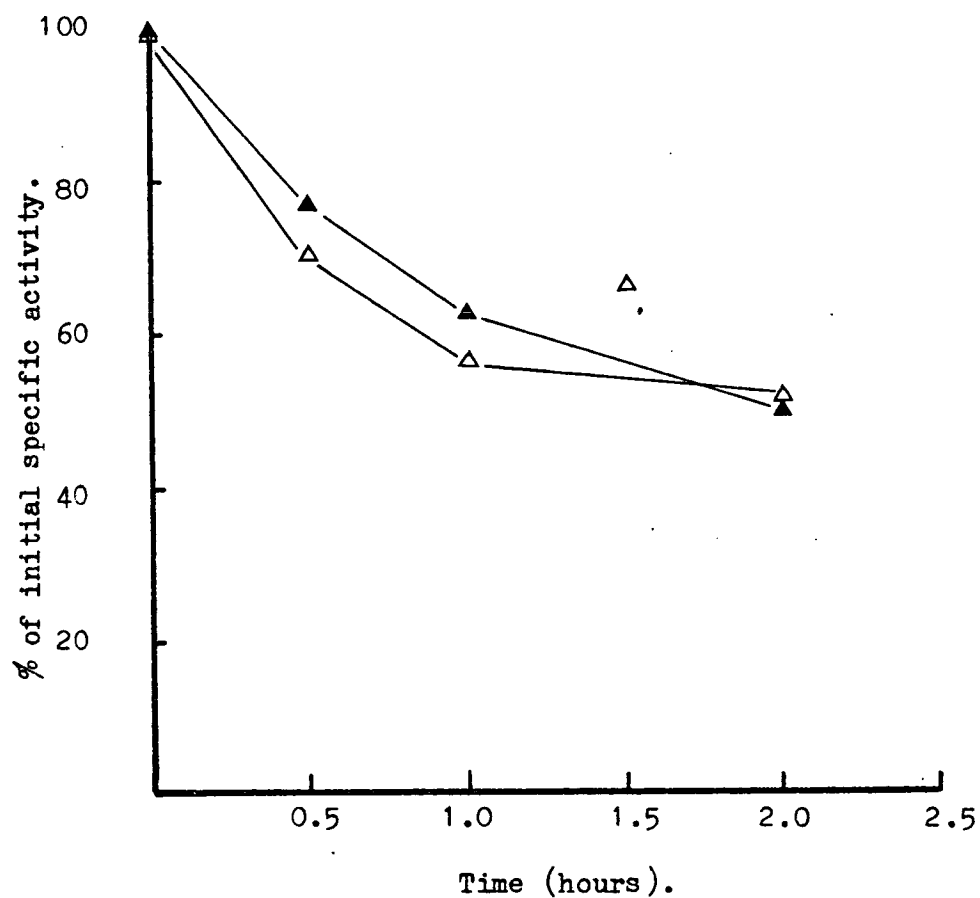


Figure 4.3

Change in activity of glutamine synthetase following addition of 7.5mM ammonium sulphate to cultures growing on glucose with 20mM glutamate. Activities are expressed as percentage of initial specific activity. ▲ S41; △ ID16D.

carbon sources which permit the best expression of the spd phenotype do not support growth of the wild-type when used as sole carbon source in minimal medium. As it was necessary to employ a minimal medium to enable comparison to be drawn with the studies on glucose minimal medium, two methods were investigated in an attempt to circumvent this problem.

Firstly it was decided to investigate the use of galactose as carbon source since earlier experiments (Table 3.1) had shown that this would support similar growth of S41 and ID16D although the latter began to sporulate on reaching stationary phase. Fast (1973) has already reported that galactose as a pre-sporulation carbon source is far less repressing to subsequent sporulation than glucose, while Polakis and Bartley (1965) have described reduced repression of Tricarboxylic acid cycle enzymes on galactose even though it is a fermentable carbon source. Therefore an effect of the spd mutation might be observed with galactose as carbon source rather than with glucose.

Arginase levels with galactose were found to be very low under each of the three growth conditions in S41 and ID16D when compared to the glucose grown control (Table 4.5). All were within the range obtained for basal levels with glucose grown cells. Glutamine synthetase levels on galactose were also found to be very low compared to those of glucose grown S41 (Table 4.6). There was on average a two-fold induction of both enzymes in all strains and the levels under each set of growth conditions was reduced as compared to the respective levels on glucose. A three to five-fold decrease was observed in the presence of ammonium ion and a six to seven-fold drop in the presence of the amino acid

Table 4.5

Arginase levels in wild-type and derepressed strains during growth on galactose as carbon source.

Growth conditions	S41 glucose	S41 galactose	ID16D galactose
+ $\text{NH}_4^+$	0.13	0.08	0.09
+ $\text{NH}_4^+$ + arginine	0.34	0.18	0.08
+ arginine	0.53	0.1	0.2

Media contained 2% carbohydrate with 7.5mM ammonium sulphate and 20mM arginine as required, cells were harvested as described in section 2.6. Samples were assayed as detailed in 2.9.1 and specific activity expressed as  $\mu\text{moles urea} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$

Growth conditions	S41 glucose	S41 Galactose	16D galactose	19D galactose
+ NH <sub>4</sub> <sup>+</sup>	0.19	0.062	0.019	0.032
+ NH <sub>4</sub> <sup>+</sup> + glutamate	0.12	0.053	0.062	0.067
+ glutamate	0.49*	0.104	0.08	0.13

Table 4.6

Glutamine synthetase levels in wild-type and sporulation derepressed strains during growth on galactose as carbon source.

Media contained 2% carbohydrate, 7.5mM ammonium sulphate and 20mM glutamate as required. Cells were harvested as described in section 2.6 and glutamine synthetase assayed as detailed in 2.9.3. Specific activity was expressed as  $\mu\text{moles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$

\* Experimentally induced low value.

as sole nitrogen source. Essentially these observations agree with the previous experiments and suggest no significant effect of the spd lesion on the repression characteristics of the enzymes tested.

Secondly, enzyme levels were followed after the transfer of cells from YEFD to YEFG. Under these conditions  $\text{NAD}^+$ -dependent glutamate dehydrogenase showed a steady decrease over the period of the experiment (Figure 4.4) but no difference could be detected between the mutant and wild-type.

The  $\text{NADP}^+$ -dependent glutamate dehydrogenase was also followed after switching from YEFD to YEFG. This enzyme, which is thought to have an anabolic function in the assimilation of ammonium ion <sup>et.al.</sup> (Dubois et.al. 1973, Middelhoven 1976), has also been proposed to have a regulatory rôle in nitrogen metabolism distinct from its enzymatic function (see Chapter 1). The absence of the enzyme leads to an insensitivity of some nitrogen repressible enzymes to ammonium ion and it is possible that an alteration in this enzyme could have an effect on sporulation. There was, however, no significant difference between the change in levels of activity in S41 and the mutant ID16D following the shift described above (Figure 4.5).

$\text{NADP}^+$ -dependent glutamate dehydrogenase minus mutants have been described (Grenson and Hou 1972) and these exhibit slower growth on minimal medium with ammonium than their parent wild-type which contrasts with the spd mutants described here whose growth rate on minimal medium with ammonium is the same as that of the parent wild-type. Thus not only is the spd phenotype incompatible with reduced specific activity of  $\text{NADP}^+$ -dependent

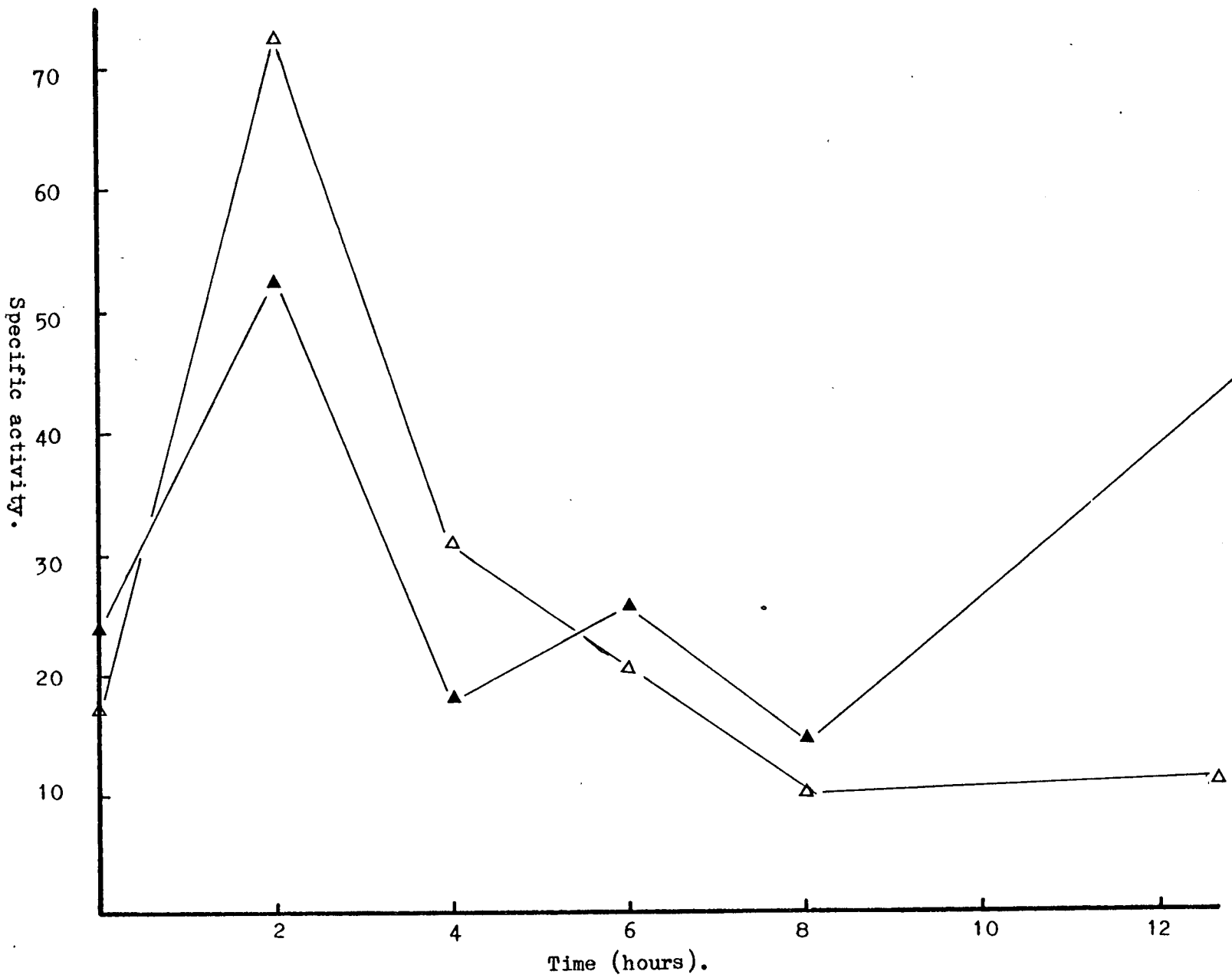


Figure 4.4

Change in levels of NAD<sup>+</sup>-dependent glutamate dehydrogenase  
(nanomoles NAD<sup>+</sup> min<sup>-1</sup> mg protein<sup>-1</sup>) following a shift from YEED to YEPG.  
Assays were carried out on sonicated extracts. ▲ S41; △ ID16D.



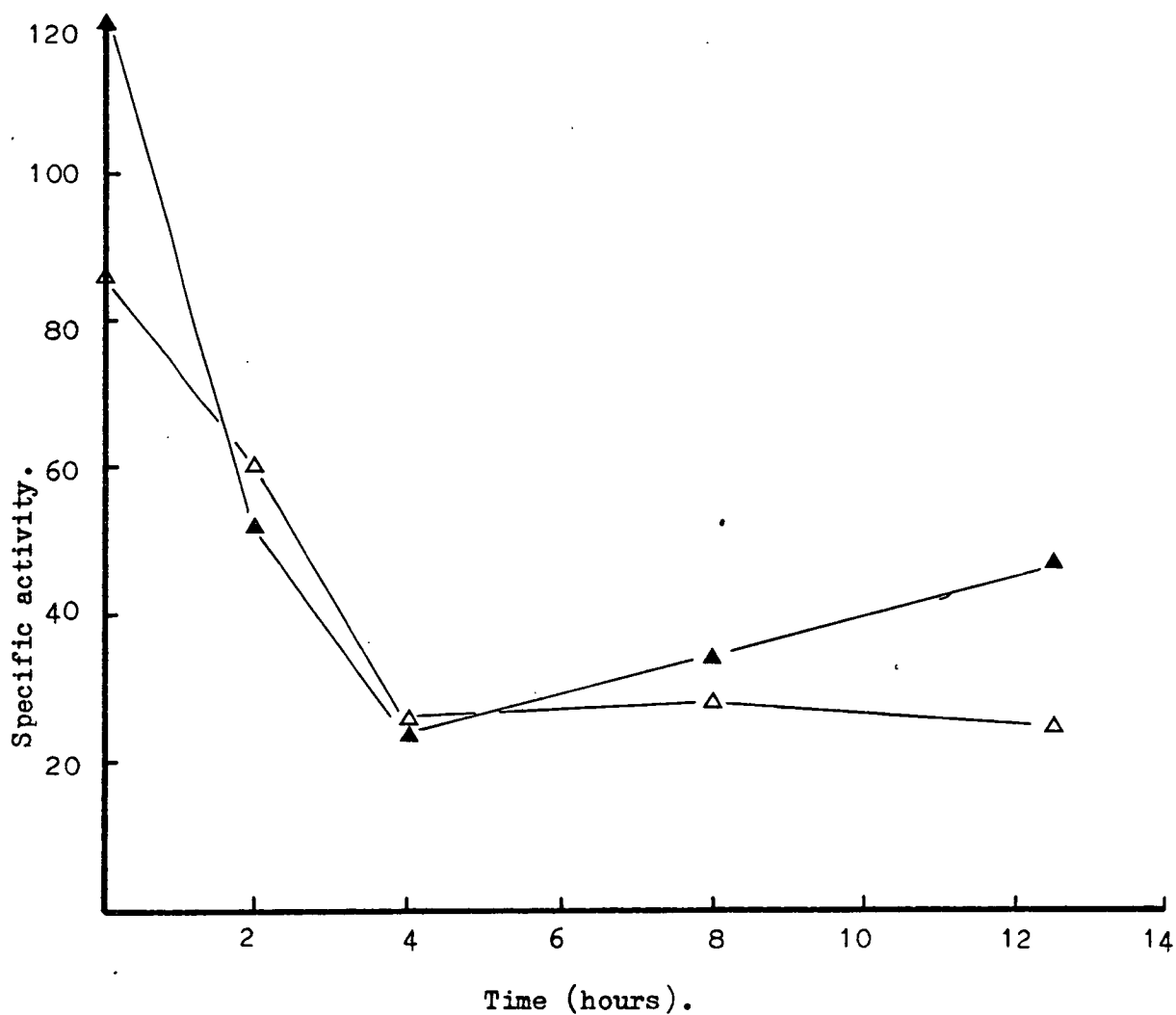


Figure 4.5

Change in activity of  $\text{NADP}^+$ -dependent glutamate dehydrogenase in S41 and ID16D following a shift from YEPD to YEPG. Specific activities are expressed as  $\text{nanomoles } \text{NADP}^+ \cdot \text{min}^{-1} \text{mg protein}^{-1}$ . Assays were carried out on cells which had been broken by sonication.

▲ S41;    △ ID16D.

glutamate dehydrogenase but also as is evident from the results presented above the spd lesion does not give rise to reduced levels of the enzyme.

## Discussion.

The absence of any effect of the spd mutation on nitrogen repressible enzyme synthesis under all the conditions studied, including those which best demonstrate the derepressed sporulation phenotype, suggests that this mutation does not affect a general site involved in nitrogen control. Thus the spd lesion does not represent a mutation similar to the areA locus in Aspergillus discussed in chapter 1, although there might be an effect of the spd mutation on enzymes not examined. This appears unlikely as they all appear to be part of different control circuits.

Glutamine synthetase has been proposed as having a regulatory role in the sporulation of Bacillus megaterium (Reysset and Aubert 1975) and in the regulation of enzyme synthesis in Klebsiella (Prival et.al. 1973) but under the conditions employed in this study there do not appear to be any alterations in the in-vivo activity of glutamine synthetase which could support its defective involvement in regulation in the spd mutants.

These conclusions raise several possibilities concerning the nature of the derepressed mutation which will be discussed in more detail in subsequent chapters but are summarised here:

- 1) The spd mutation is in a locus specific to the control of sporulation in which event there are two alternatives:
  - a) The nitrogen regulation of sporulation may be reduced or absent in spd mutants. Therefore when the carbon source is one which does not repress functions required for sporulation the specific nitrogen control, which determines whether growth or sporulation will occur, is defective and the only alternative for the cell is to sporulate.

b) A repressor of sporulation fails to be produced during growth on non-repressing carbon sources. This in its simplest form is the less likely as this would necessitate a carbon control only on sporulation and there is ample evidence to show susceptibility of sporulation to nitrogen compounds. A more complex system involving regulatory interaction between carbon and nitrogen controls could be included in this but there is insufficient evidence to build such a model at this juncture. The studies on nitrogen regulation in Aspergillus, however, do indicate some form of interaction between carbon and nitrogen repression although even here further study is required (see chapters 1 & 8; Arst and Cove 1973).

2) Alternatively the spd phenotype could derive from a mutation producing a primary defect in carbon metabolism. This could in turn have a secondary effect on nitrogen uptake and/or metabolism hence producing a starved or semi-starved condition which induces sporulation.

#### Effect of carbon source on nitrogen-repressible enzyme synthesis.

In this study lower levels of glutamine synthetase and arginase were observed during growth on galactose compared to those on glucose and the increase in activity when glutamate or arginine were present as sole nitrogen source was also lower with galactose as carbon source. This latter effect was also observed by Middelhoven (1970) although he did not report an overall reduction in arginase levels. As his strain appeared to grow on minimal ethanol and lactate media, whereas ours do not, it may be that strain differences are responsible for the observed anomalies.

The results obtained in this study raise the possibility that the rate of synthesis of certain enzymes may be dependent on growth rate, thus at the lower growth rates less enzyme activity is required to provide sufficient nitrogen to maintain balanced metabolism. Therefore the growth rate on galactose with amino acid is such that only enzyme levels associated with growth on glucose with ammonium ion are necessary to incorporate sufficient nitrogen for growth.

Chapter 5.NITROGEN SOURCE EFFECTS ON SPORULATION

From the results presented in chapter three and from the literature (Miller 1963) it was suggested that relief of nitrogen source repression plays an important part in initiation of sporulation. The spd1 mutation may alter the nitrogen repression control of sporulation and therefore should be useful for study of the effects of different nitrogen sources on sporulation.

While the effect of different nitrogen sources has been studied by a number of workers there has been little or no attention paid to the effects of different concentrations of nitrogen sources. In particular this is most noticeable with ammonium ion which Miller (1963) reported to be inhibitory to sporulation at 10mM which is in excess of the ammonium sulphate concentration in our minimal growth medium. Therefore there are no data in the literature to indicate whether sporulation is particularly sensitive to any one nitrogen source at low concentrations.

Therefore in this chapter the susceptibility of sporulation to repression by different nitrogen sources at various concentrations has been studied in our wild-type and sporulation derepressed strains, which may be altered in their sensitivity to nitrogen repression, in order to determine whether ammonium ion or other nitrogen compounds is the primary effector of repression. To this end also non-metabolisable analogues of ammonium ion have been used in an attempt to further distinguish between the two possible mediators of repression.

Inhibition of sporulation using various concentrations of ammonium sulphate.

The effect of ammonium ion was examined by counting sporulation frequencies in cultures to which various concentrations of ammonium sulphate were added at  $t_0$  to the sporulation medium. S41 wild-type and spd1 mutants ID16D, ID19D and ts85 were compared in the expectation that the mutants would show greatly reduced sensitivities to ammonium ion over the concentration range employed. It was also thought probable that a range of different sensitivities might be observed among the spd1 mutants themselves, reflecting variable effects of the different alleles. Strains were transferred from early stationary phase YEPD grown cultures to 1% potassium acetate as described in section 2.6.

Strain S41 24 hours after resuspension in sporulation medium showed a steady decrease in sporulation which was inversely proportional to the ammonium sulphate concentration over the 0.2-1.0mM range (Figure 5.1). Above 2.0mM sporulation was absent (Table 5.1). By 48 hours, however, this inhibitory effect of ammonium sulphate between 0.2-1.0mM was no longer apparent, or very much reduced (Figure 5.1). Cultures subjected to concentrations above 2.0mM showed no release of the inhibitory effect with time. Subsequent plating of samples from 10mM ammonium sulphate onto YEPD indicated that there had been a very substantial decrease in viability.

Derepressed mutant ID16D showed a similar response to s41 in the 2-10mM range. However, a major difference was apparent over the 0.2-1.0mM range 24 hours after resuspension (Figure 5.1). Here there was no significant inhibitory effect of ammonium

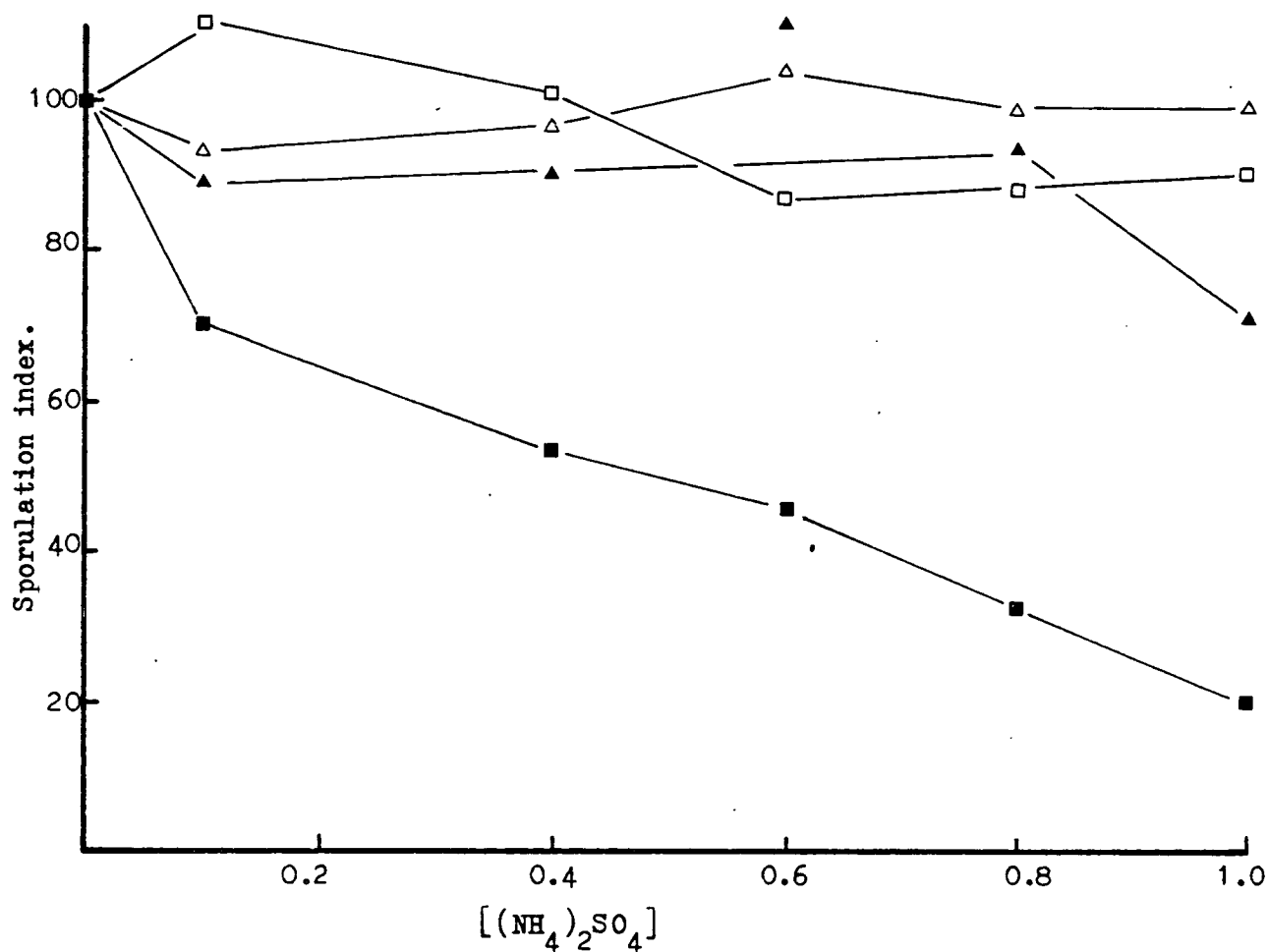


Figure 5.1

Effect of addition of various concentrations of ammonium sulphate to sporulation medium (1% potassium acetate, arginine  $20\mu\text{g.ml}^{-1}$ ) at time zero. The control contained no ammonium sulphate. Strains were resuspended to similar turbidities. Sporulation is expressed as percentage of the control sporulation frequency (taken as 100%) at that time.

■ S41 24 hours; □ S41 48 hours; ▲ ID16D 24 hours; △ ID16D 48 hours.



Table 5.1 Appearance and sporulation index of strains in high concentrations of ammonium sulphate.

Strain	2.0mM	5.0mM	10mM
S41	5-7, considerable cell lysis and phase dark cells.	0, increasing nos. of phase dark cells	0, almost all cells phase dark.
ID16D	4-6, "	0, "	0, "
ID19D	3-11, "	0, "	0, "
ts85	3-8 "	0, "	0, "

sulphate on sporulation as compared to the control containing none. There was a slight inhibitory effect of 1.0mM ammonium sulphate observed, but this was very small compared to that found in the wild-type. Therefore ID16D appears to have reduced sensitivity to ammonium sulphate only over the range 0.2-1.0mM under these conditions.

The concentration of ammonium sulphate in minimal growth medium is 7.5mM. When a similar concentration is added to sporulation medium inhibition of sporulation in the mutant is complete. The other two spd mutants tested, ts85 and ID19D, showed slightly different responses. ID19D while similar to ID16D appeared to be more sensitive (Figure 5.2) and ts85 was as sensitive as the wild-type control (Figure 5.3). All three have been shown to be alleles of the same gene (I.W.Dawes pers.comm.). All four strains showed considerable tendency to clump in high concentrations of ammonium sulphate and an increasing frequency of phase dark cells was observed with increasing concentrations (Table 5.1). S41 had a tendency to exhibit pseudo-mycelial growth patterns of long vacuolated, cells on 10mM ammonium sulphate. ID16D in the same circumstances though equally prone to clumping, consisted of round non-vacuolated, cells (Table 5.1).

Sporulation appears to be very sensitive to ammonium sulphate; concentrations which support growth when a suitable carbon source (e.g. glucose) is present are extremely toxic to cells which are in a non-growth / developmental situation. spd1 mutants show no resistance to high concentrations of ammonium ion but ID16D and ID19D are resistant over the 0.2-1.0mM range. Sporulation in the wild-type over this range is merely delayed. By 48 hours after

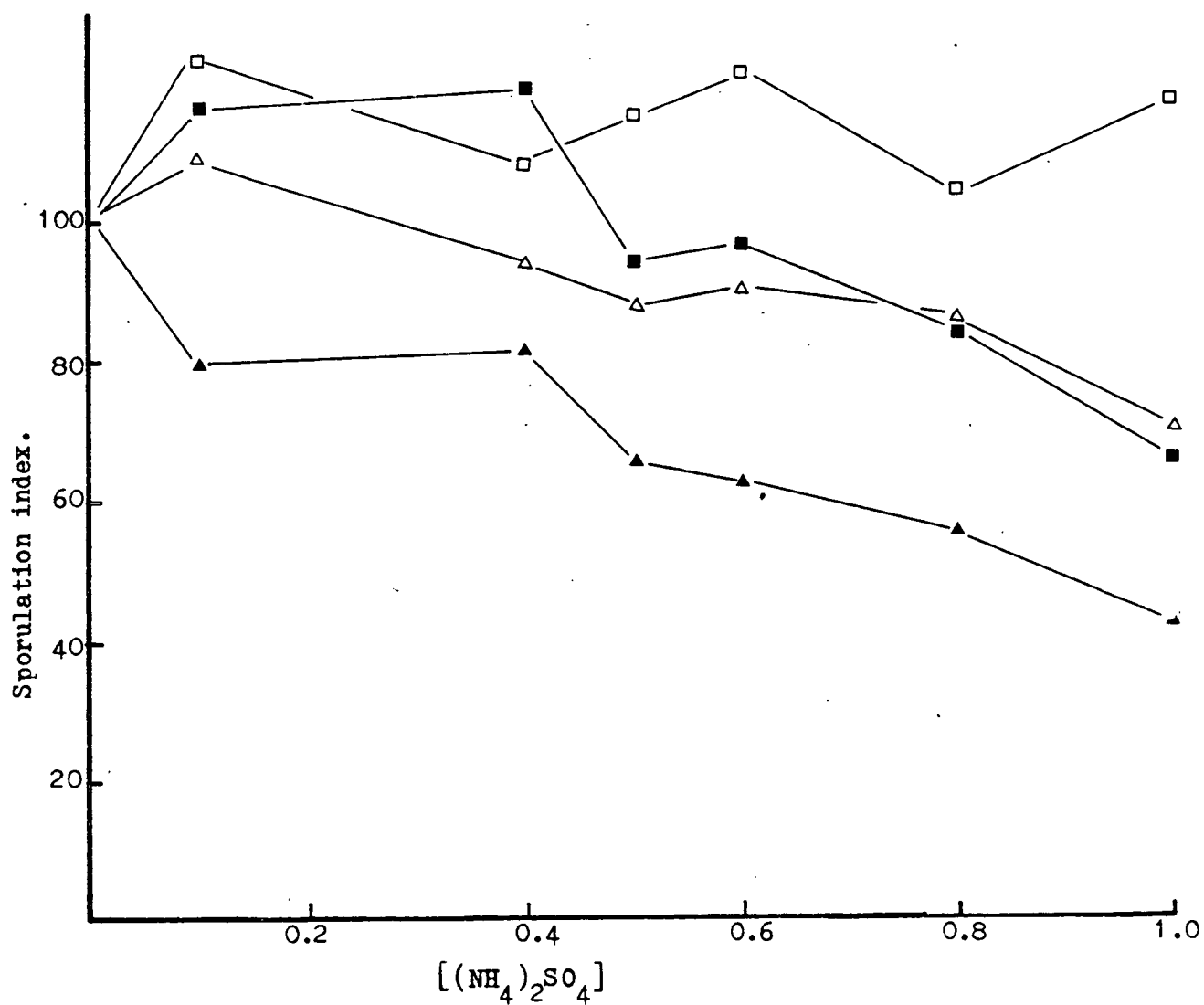


Figure 5.2

Sporulation obtained in S41 and ID19D (spd1-2) in sporulation medium containing various concentrations of ammonium sulphate.

Protocol as in figure 5.1

■ 24 hours ID19D; □ 48 hours ID19D; ▲ 24 hours S41; △ 48 hours S41

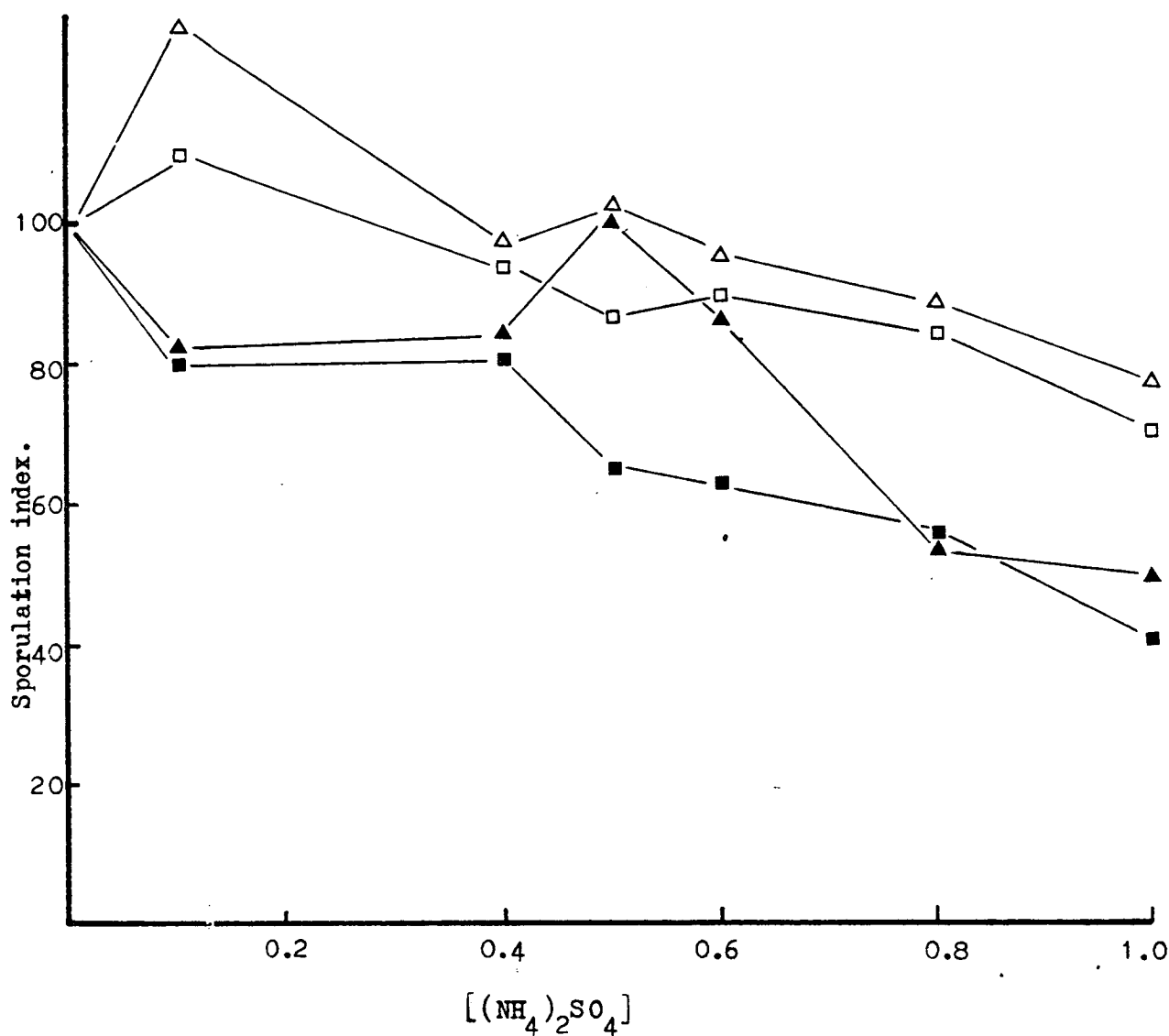


Figure 5.3

Sporulation obtained in S41 and ts85 (spd1-4) after resuspension in sporulation medium with various concentrations of ammonium sulphate. Protocol as in figure 5.1.

▲ 24 hours ts85; △ 48 hours ts85; ■ 24 hours S41; □ 48 hours S41.

resuspension in sporulation medium sporulation showed a similar pattern in S41 and ID16D.

It seems probable therefore that sporulation is delayed in S41 over the time it takes for the cells to reduce exogenous ammonium ion concentration by incorporating it into cellular material. This was tested in part using Nessler's reagent. The results though not conclusive indicate in both S41 and ID16D by 24 hours the original 1.0mM concentration of ammonium sulphate had been considerably reduced but not completely used up. However, excretion of ammonium ion from the cells could account for this positive reaction. Ramirez and Miller (1964) suggested that some of the intracellular amino acids are oxidised during sporulation which leads to the appearance of ammonium ion in the sporulation medium.

#### Amino acids as repressors of sporulation.

The sporulation derepressed mutants were originally recovered from a selection régime containing a mixture of yeast extract and peptone as nitrogen sources. The available nitrogen is mainly in the form of amino acids and peptides. Tests with Nessler's reagent indicated that the ammonium ion concentration is very low and that formed by breakdown of amino acids during autoclaving is probably used rapidly by the growing cells.

Miller (1963) obtained inhibition of sporulation with both ammonium ion and amino acids but, as considered in the introduction, there is a degree of controversy over the relative effectiveness of ammonium ion as opposed to amino acids as mediators of repression of enzyme synthesis. In view of this, it was decided to test various amino acids, some readily utilisable as nitrogen sources, others

not, for their ability to repress sporulation, both in wild-type and spd1 mutants. While it has already been established that ammonium ion has a strong inhibitory effect on sporulation even in the spd1 mutants at relatively low concentrations this does not necessarily exclude the possibility that ammonium ion exerts its effect through metabolism to amino acids. Different single amino acids were therefore tested at different concentrations to determine if any one amino acid or several from one group could act efficiently as repressors of sporulation.

Initially amino acids were added at 3.0mM since ammonium sulphate has a considerable inhibitory effect between 1 and 2mM. From Table 5.2 it can be seen that arginine, glutamine and glutamate far from inhibiting sporulation actually had a slight promoting effect in both wild-type and derepressed strains. Ammonium sulphate at 1.0mM produced the same sort of strong inhibition observed previously (Figure 5.1) in S41, ID16D being relatively resistant at 24 hours. Therefore there appears to be no inhibitory effect of 3mM arginine, glutamine or glutamate on sporulation in S41 and/or ID16D. Moreover, ID16D appears to be neither more nor less sensitive than S41.

#### Effect of 10mM amino acids on sporulation.

Amino acids have been used at 0.1% (w/v) to repress enzyme synthesis (Bossinger et.al. 1974). This represents <sup>approximately</sup> two times the concentration used in the present experiments (3.0mM). Bossinger et.al. (1974) also suggested that amino acids capable of undergoing transamination reactions such as glutamine and serine (i.e. capable of acting as mediators of amino group transfer in biosynthetic pathways), were more effective repressors of

Table 5.2 Sporulation in the presence of  $\text{NH}_4^+$ ,  $\text{Cs}^+$  and various amino acids at 3.0mM.

Addition	S41		ID16D	
	22 hrs	38hrs	22hrs	38hrs
None	100	100	100	100
1.0mM $(\text{NH}_4)_2\text{SO}_4$	2	8	56	70
2.0mM CsCl	97	100	117	125
Arginine	127	94	98	121
Glutamine	113	94	122	121
Glutamate	103	114	97	116

Cells were grown to early stationary phase in YEPD medium and resuspended at zero time in 1% potassium acetate containing the stated additions.

enzyme synthesis. With this in mind a more complete range of amino acids was tested for their effects on sporulation at 10mM. This concentration permits good growth when a readily utilised amino acid is present as sole nitrogen source.

Again, however, similar observations were made. Only glutamine and histidine produced any significant decrease in frequency of sporulation in the wild-type S41 (Table 5.3). (note: glutamine when autoclaved produced a complete inhibition of sporulation. This was due to considerable hydrolysis to yield ammonium ion since a strong positive reaction was obtained using Nessler's reagent on autoclaved glutamine but not after filter sterilisation.) The inhibitory effect of 10mM glutamine was by no means complete; a sporulation index of 67 at 24 hours was observed in the wild-type. From this it can be suggested that glutamine inhibits sporulation in just under half the cell population and that this effect is maintained with time unlike that with 1.0mM ammonium sulphate.

Glutamine is capable of considerable interconversion with free ammonium ion and glutamate and it is therefore surprising that it does not produce a much more marked repression of sporulation, especially considering that glutamine hydrolysed by autoclaving is completely inhibitory. The most likely conclusion from this is that glutamine itself has only a very small inhibitory effect on sporulation and any repression observed is due to its acting as a source of ammonium ion. This result also suggests the interconversion of glutamine with glutamate and ammonium ion must be regulated in such a way as to maintain a low pool of free ammonium ion during sporulation.



Table 5.3 Effect of 10mM amino acids on sporulation in S41 and ID16D. Sporulation index is expressed as % of control sporulation frequency.

Amino acid	S41		ID16D	
	24 hrs	48 hrs	24 hrs	48 hrs
Control	100	100	100	100
1.0mM $(\text{NH}_4)_2\text{SO}_4$	53	60	79	62
Arginine	122	93	141	109
Methionine	82	78	115	94
Glutamate	102	101	118	85
Glutamine	67	52	91	48
Serine	116	88	138	91
Proline	106	79	126	79
Histidine*	83	79	62	67
Lysine*	73	67	79	64

\*relatively insoluble

ID16D showed a lower sensitivity to 10mM glutamine at 24 hours than did S41 (sporulation index of 91 at 24 hours). No other significant inhibition by any other amino acid except histidine was observed. As was found for strain S41 this inhibitory effect of glutamine was not overcome with time.

Effect of analogues of  $\text{NH}_4^+$  on sporulation in S41.

In view of the previous results, it appeared that sporulation is extremely sensitive to low concentrations of  $\text{NH}_4^+$  but insensitive to fairly high concentrations of single amino acids. This raises the possibility that  $\text{NH}_4^+$  is the mediator of repression and does not require to be metabolised to other compounds.

Caesium chloride and methylammonium chloride have been used successfully as analogues of  $\text{NH}_4^+$  in Aspergillus (Arst and Cove 1973). Roon et.al (1975), have established that methylamine (ion) is not metabolised further, and has a high affinity for the  $\text{NH}_4^+$  transport system in Saccharomyces. At low concentrations, 1-4mM, it has been established that  $\text{Cs}^+$  and methylamine do not inhibit growth in our strains (I.W. Dawes pers. comm.)

Preliminary results using  $\text{Cs}^+$  as an analogue indicated that  $\text{NH}_4^+$  may have to be metabolised in order to produce inhibition, as no repressive effect of 2mM caesium chloride was observed in our wild type S41 or derepressed strain ID16D (Table 5.2). Subsequent results also showed no inhibitory effect of  $\text{Cs}^+$  but using methylammonium chloride strong inhibition was obtained (Table 5.4). The inhibitory effect of 4mM methylammonium chloride was roughly equivalent to that produced by 2mM  $(\text{NH}_4)_2\text{SO}_4$  as might be expected. This suggests that methylamine can act as an analogue of  $\text{NH}_4^+$  in this strain. It also further indicates that  $\text{NH}_4^+$  itself acts as an inhibitor of sporulation and that metabolism to other compounds is not required. It would appear that caesium chloride at these

Table 5.4 Effect of the  $\text{NH}_4^+$  analogues caesium chloride and methyl ammonium chloride on sporulation in S41, wild-type.

	24 hours		48 hours	
	% Sporulation	Sporulation index	% Sporulation	Sporulation index
1% potassium acetate	57	100	66	100
2mM $(\text{NH}_4)_2\text{SO}_4$	1	2	0	0
2mM CsCl	42	73	60	91
4mM CsCl	55	96	60	91
2mM methyl ammonium chloride	36	63	52	79
4mM methyl ammonium chloride	6	10	4	6

concentrations does not act as an analogue in yeast. The complete lack of an effect suggests that it does not permeate the cells.

It is perhaps worth noting that many of the tetrads formed in low concentrations of methylemine had a rather abnormal appearance. Although recognisable they were smaller and less refractile than controls. As with 2mM ammonium sulphate there were many phase dark cells in 4mM methylammonium chloride.

Attempts to mimic the spd phenotype in the wild-type.

Derepressed strain ID16D (spd1-1) grows poorly in YEP-glycerol medium and as spores are first noticeable in 24 hours after resuspension in glycerol containing medium it is conceivable that a proportion of the population initiates at once and that subsequent stages in the sporulation sequence are insensitive to any environmental inhibitors. If this latter surmise is correct, it should be possible to produce a phenocopy of the spd1 mutants in the wild type strain S41 by initiating sporulation in 1%w/v potassium acetate and transferring the culture back to YEP-glycerol.

Cells were pregrown in YEP-glycerol so that they would already be adapted to a non-fermentative type of metabolism. Logarithmically growing cells were transferred to potassium acetate and samples removed at various times and replaced in YEP-glycerol. The hypothesis that once sporulation has been initiated by a pulse treatment with potassium acetate it can continue following resuspension in YEP-glycerol is in fact testing whether initiation is the main factor governing progress of sporulation conditions in which derepressed strains sporulate. However, as can be seen from the table (5.5) no sporulation was observed in any of the samples taken from potassium acetate and subsequently incubated for 24 hours

Table 5.5 Sporulation capacity of S41 after resuspension in YEPG following exposure of a log phase YEPG-grown culture to sporulation medium for varying lengths of time.

Time spent in sporulation medium (hours)	% sporulation in YEPG 24 hours after resuspension from potassium acetate.	% sporulation in YEPG 4 days after resuspension from KAc	Growth on YEPG
0.5	0	0.5	++
1.0	0	15	++
2.0	0	5	++
4.0	0	7	++

A log phase YEP-glycerol culture of S41 was centrifuged and washed once with 0.5M sodium phosphate pH 7.7 and resuspended in 1% potassium acetate with arginine. Samples of this culture were taken at the time intervals above and resuspended in YEPG.

in YEP-glycerol. Even after four days in YEP-glycerol, there was no significant correlation between time spent in potassium acetate and the percentage sporulation.

Therefore, the wild-type strain S41, even though initiated, remains sensitive to environmental stimuli of this nature at least over the first four hours following resuspension in potassium acetate. It also follows that the derepressed mutant ID16D is less sensitive to these same factors both during initiation and subsequent development, and since the spd1-1 phenotype is the result of a single mutation, it may be altered in the mechanism controlling both the process of initiation and that of subsequent development over the early stages.

#### Discussion.

$\text{NH}_4^+$  has been found to completely repress sporulation at concentrations in excess of 2mM ammonium sulphate, whereas exogenous amino acids added singly at 10mM concentration do not. This includes glutamate and to some extent glutamine, and since glutamate is an intermediate in the major route for the assimilation of exogenous  $\text{NH}_4^+$  it seems likely that  $\text{NH}_4^+$  is responsible for the repression of sporulation.

This view is confirmed by the use of a non-metabolisable analogue of  $\text{NH}_4^+$ , methylamine, which can repress sporulation at similar concentrations to that of  $\text{NH}_4^+$ . Although its repression effects may not be simple, it seems that they are not due to toxicity as cells grow in the presence of methylamine at these low concentrations.

Derepressed mutants ID16D and ID19D are less sensitive to ammonium sulphate at concentrations of 1mM and below, than is the

wild-type. Above 2mM ammonium sulphate inhibits sporulation in both mutants and wild-type and leads to loss of viability under sporulation conditions. This effect was also observed by Pinón (1977). While other interpretations are possible, it seems likely that the spd1 lesion may affect a control system regulating the initiation of sporulation. This system is sensitive to  $\text{NH}_4^+$  at levels below 2mM in the wild-type. Above 2mM,  $\text{NH}_4^+$  exerts another effect which may be at or near initiation but also possibly later in the sporulation sequence (Pinón 1977; Durieu-Trautmann and Delavier-Klutcho 1977).

Since sporulation seems to be extremely sensitive to low levels of  $\text{NH}_4^+$ , it is probable that the intracellular  $\text{NH}_4^+$  monitoring mechanism for regulation of nitrogen repressible enzyme synthesis is different from that of sporulation. Supporting this is the lack of an effect of the spd1 lesion on nitrogen-repressible enzyme synthesis.

The fact that the wild-type does sporulate, but much more slowly (a reduced frequency of asci being observed at 24 hours) than the derepressed mutants in 1mM ammonium sulphate favours the view that in the presence of  $\text{NH}_4^+$  sporulation is initiated with a low probability. A mutation affecting the affinity of the repression system for  $\text{NH}_4^+$  would increase the rate of initiation of sporulation. Different alleles at the spd1 locus would therefore be expected to show different responses to  $\text{NH}_4^+$  at concentrations below 2mM.

On this basis, it should be possible to select mutants which are resistant to  $\text{NH}_4^+$  at concentrations less than 2mM. These may be allelic to spd1. From these, double mutants could be sought which sporulate at concentrations greater than 2mM. Similarly, mutants which are defective in  $\text{NH}_4^+$  transport should display hypersporulation on non-repressing carbon sources.

The lack of inhibition by amino acids at such high concentrations is surprising as many, for example arginine, can supply much more nitrogen mole for mole than can  $\text{NH}_4^+$  and, at the concentration used here, (10mM), can support growth. Despite the results it seems unlikely that amino acids can exert no repression of sporulation. In fact if this were so one might expect the wild-type strain S41 to sporulate rather than grow in a medium containing YEP and a non-repressing carbon source. It seems more likely that amino acids represent a rather weak form of repression, only being effective at very high concentrations or as a rich mixture as is present in yeast extract bactopectone.

These studies on the effects of  $\text{NH}_4^+$  and amino acids on sporulation have been confirmed by Pinõn (1977). He obtained similar effects by  $\text{NH}_4^+$  over the same range of concentrations and also observed a failure of exogenous amino acids to inhibit sporulation. He did however obtain severe repression using glutamine which was not observed in this laboratory.

These results however do not rule out the possibility that a defect in metabolism of non-fermentable carbon sources could have a pleiotropic effect on uptake and utilisation of certain nitrogen sources. This, in theory, could produce an spd phenotype. It may be that under conditions where the carbon source is poor  $\text{NH}_4^+$  is more readily taken up than are exogenous amino acids; this would be reflected in a poor repression of sporulation by individual amino acids.

The ability of spd1 mutants to transport and metabolise non-fermentable carbon sources is considered in the next chapter.



## Chapter 6.

### REGULATION OF CARBON AND ENERGY SOURCE METABOLISM IN WILD TYPE AND DERERESSED STRAINS.

Superficially one obvious explanation of part of the spd phenotype is that failure to grow on certain carbon sources results from a defect in either their transport or metabolism. Therefore examination of uptake of these carbon sources and the regulation of carbon repressible enzyme synthesis is essential to an understanding of the spd phenotype.

In the past mutants have been isolated which are unable to grow on a wide variety of carbon sources. For example, mutations affecting pyruvate kinase preclude growth on glucose, glycerol and galactose as carbon sources but not with lactate or acetate (Sprague 1977).

A single mutation leading to failure to produce isocitrate lyase and fructose-1,6- diphosphatase leads to an inability to grow on gluconeogenic carbon sources (Zimmermann et.al. 1977). Petite mutations do not permit growth on non-fermentable carbon sources, neither do they sporulate (Ephrussi 1953). However, apart from this latter case it is not known whether any other mutations affecting carbon metabolism permit sporulation to occur.

From chapter 3 it can be seen that sporulation cannot proceed efficiently in the absence of a carbon source. It therefore follows that any defect in carbon metabolism of the spd mutants cannot be one which completely blocks utilisation of a particular carbon source, nor alternately, affect some area of metabolism essential to sporulation. The range of carbon sources whose

utilisation for growth is affected by the single spd1 mutation indicates that the mutants must either be altered in an enzymatic step common to metabolism of all these carbon sources or in a common regulatory function.

In this chapter the uptake and incorporation of carbon sources on which the spd mutant ID16D does not grow and its ability to undergo derepression of certain carbon repressible enzymes involved in metabolism of these carbon sources are studied. In addition a study of one of the possible means of regulation, that mediated by cAMP, has been carried out bearing in mind that E.coli mutants defective in adenyl cyclase have undetectable levels of cAMP and a pleiotropic inability to grow on many carbon sources (Perlman and Pastan 1969).

Uptake of  $^{14}\text{C}$  labelled carbon sources into wild-type and sporulation derepressed strain ID16D.

Uptake and incorporation of radioactively labelled substrates ( $^{14}\text{C}$  glycerol and acetate) were studied using 60% ethanol to separate ethanol soluble precursor pools from insoluble, macromolecular, material. Use of 60% ethanol, as opposed to the customary trichloroacetic acid pool extraction procedure allowed samples to be processed more rapidly and no lengthy incubation on ice was required.

Following transfer of logarithmically growing cells from YEPD to a five fold dilution of YEP-acetate medium containing  $^{14}\text{C}$  acetate at  $1.0\mu\text{Ci.ml}^{-1}$  uptake of acetate into cells and its incorporation into macromolecules were found to be similar (Figures 6.1 and 2).

Initial rate of uptake into pool material was the same in S41 and ID16D (Figure 6.1) but there appeared to be, in the spd mutant, a slightly higher steady pool size than that in the wild-type. This could indicate that incorporation into macromolecular material may be slightly reduced in the mutant. However, it can be seen from Figure 6.2 that the rate of incorporation into ethanol insoluble material was the same for both mutant and wild-type, although ID16D appeared to lag behind the wild-type in establishing a linear rate. The changes in turbidity measured throughout the course of this experiment indicated that ID16D showed a poorer growth response when compared with S41. Overall this result establishes that acetate uptake and incorporation are not substantially altered in ID16D. It seems unlikely that the small reduction noted in incorporation could account for the

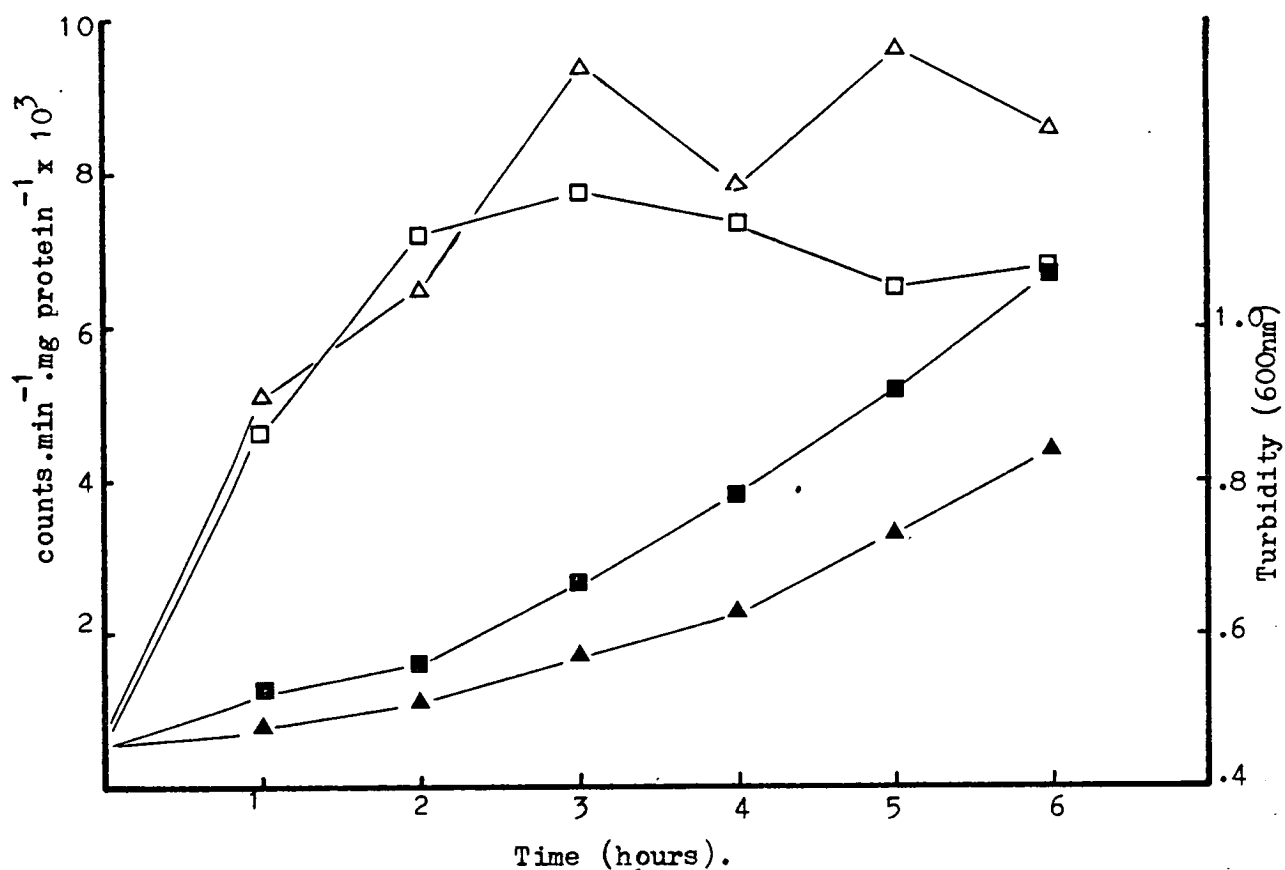


Figure 6.1

Uptake of  $^{14}\text{C}$  acetate into ethanol soluble material in a five-fold dilution of YEP-acetate.

□ S41; △ ID16D.

Change in turbidity during the coursed of the experiment.

■ S41; ▲ ID16D.

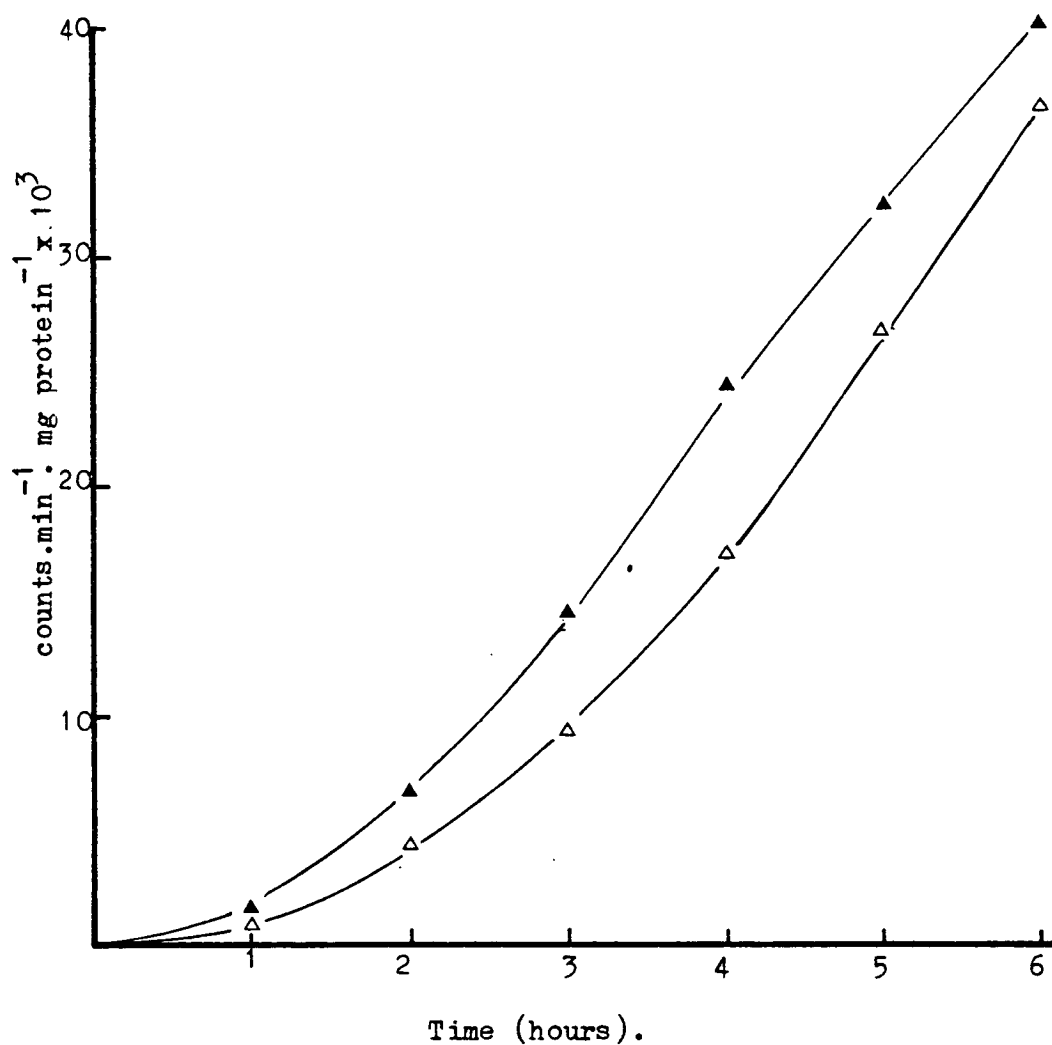


Figure 6.2

Incorporation of  $^{14}\text{C}$  acetate into ethanol insoluble material in a five-fold dilution of YEP-acetate.

▲ S41; △ ID16D. ( Label at 1.0 uCi/ml )

"decision" to sporulate rather than divide.

Studies on the uptake of  $^{14}\text{C}$  glycerol yielded similar results. Again the pool size was slightly higher in ID16D than in S41 but this small difference was not reflected in incorporation into macromolecular material (Figures 6.3 and 4). Again there was no apparent difference in rate of label accumulation between S41 and ID16D over eight hours.

Rate of accumulation of label over a short time period (10 min) in cells which had been incubated in a five fold dilution of YEP-glycerol for five hours was also examined (Figure 6.5). These rates were found to be similar in both wild-type and mutant.

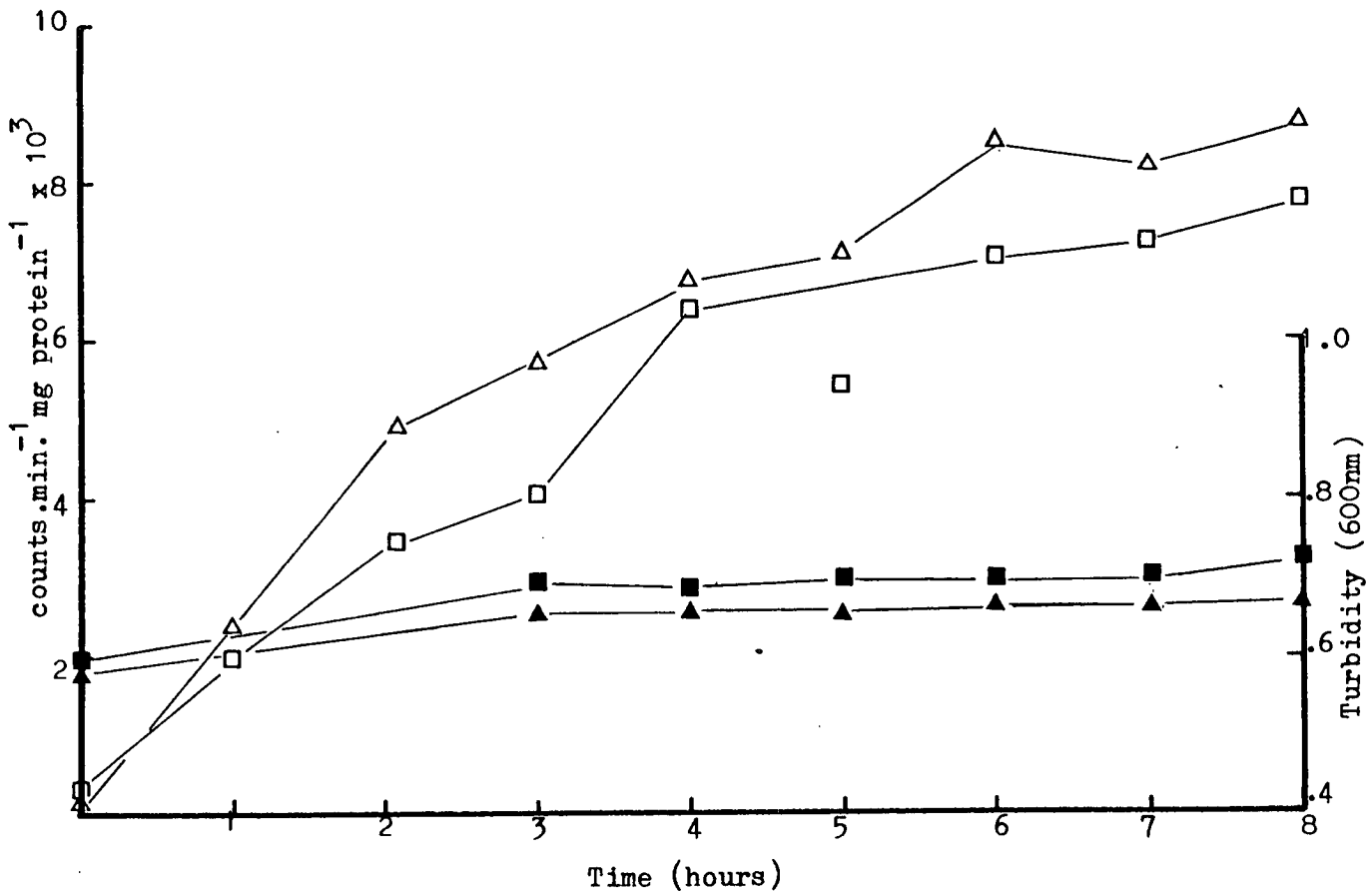


Figure 6.3

Uptake of  $^{14}\text{C}$  glycerol into ethanol soluble material and change in turbidity during the course of the experiment in S41 and ID16D.

□ S41; △ ID16D; uptake into ethanol soluble material.

■ S41; ▲ ID16D; turbidity at 600nm.

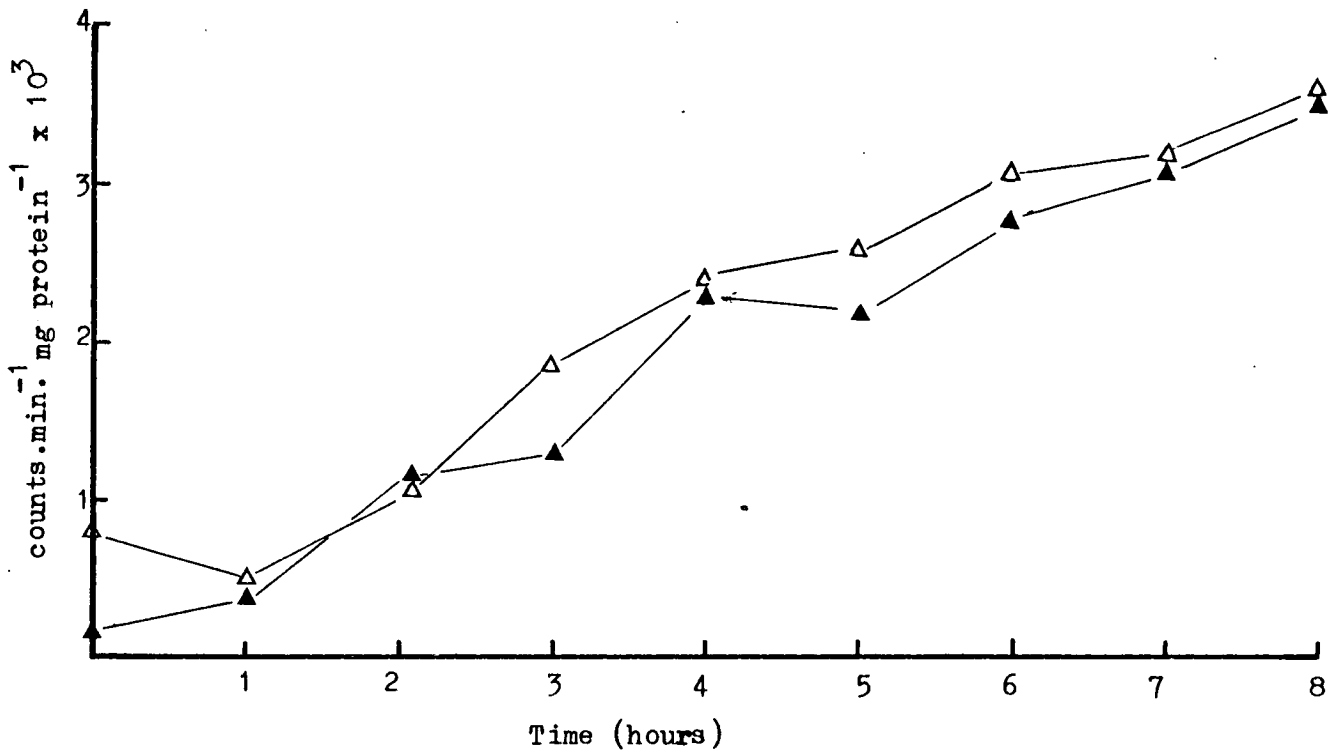


Figure 6.4

Incorporation of  $^{14}\text{C}$  glycerol into ethanol insoluble material in   ▲ S41 and   △ ID16D.



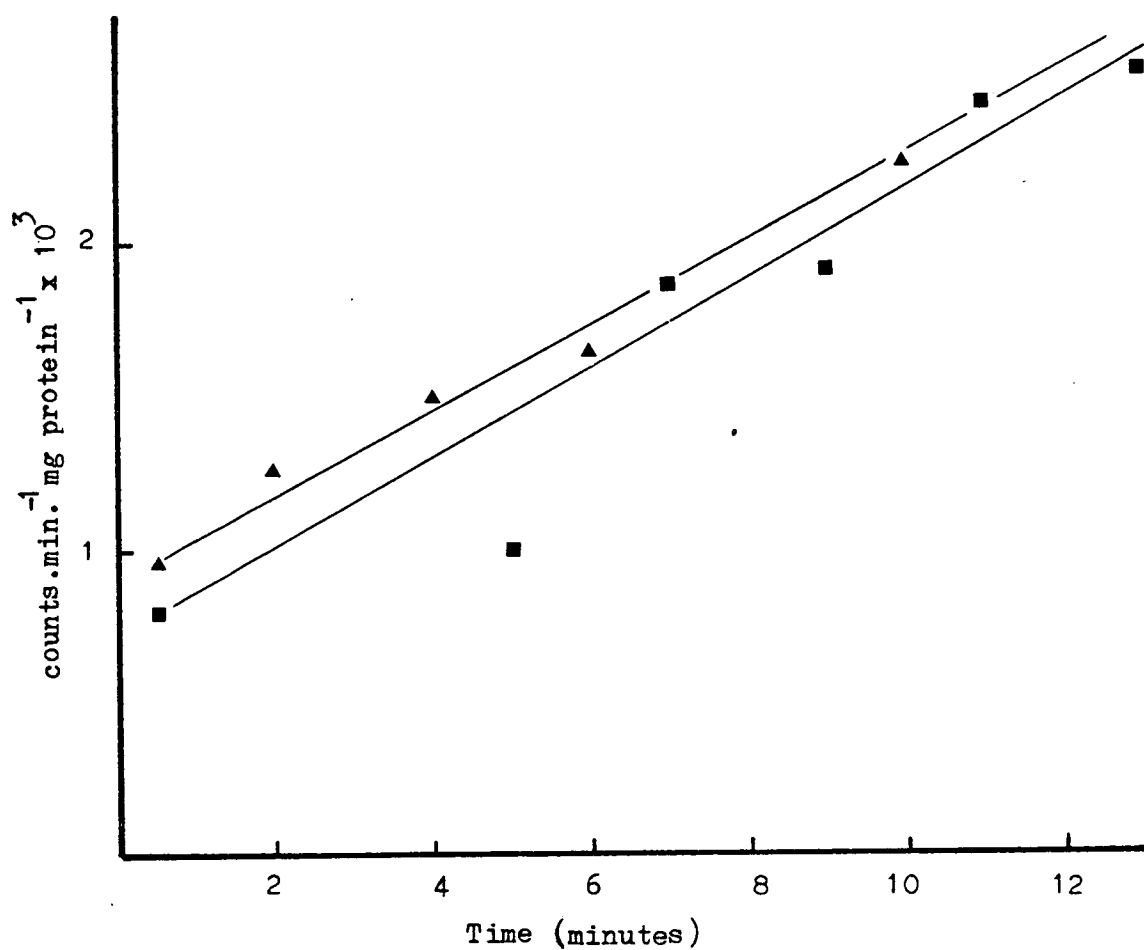


Figure 6.5

Rate of incorporation of  $^{14}\text{C}$  glycerol into S41 and ID16D over a ten minute period five hours after resuspension in a five-fold dilution of YEP-glycerol. ■ S41; ▲ ID16D.

### Glycerol catabolism in wild-type and sporulation derepressed strains.

In Saccharomyces catabolism of glycerol has been shown to proceed via glycerol 3-phosphate to dihydroxyacetonephosphate through the action of glycerol kinase and glycerol 3-phosphate dehydrogenase respectively. The synthesis of these enzymes has been shown to be subject to catabolite repression by glucose (Sprague and Cronan 1977).

Analysis of the derepressed levels of these enzymes in our strains showed no significant difference between the spd1 mutant ID16D and the wild-type S41. Samples were taken five hours after transfer of logarithmically growing cells from YEPD to YEPG, by which time the wild-type adapts to growth on glycerol (Figure 3.3) and levels at  $t_0$ , representing those for exponential growth on glucose were also similar in S41 and ID16D. (Tables 6.1 and 6.2).

The enzyme levels obtained for both glycerol kinase and glycerol 3-phosphate dehydrogenase were lower than those obtained and Cronan (1977). This may be attributed to strain variation or differences in treatment of cells prior to assay.

The main point to be noted therefore is that the spd1 mutant behaved similarly to the wild-type and thus the inability of the mutant to use glycerol for growth in complex media cannot be due to insufficiency in the levels of glycerol kinase or glycerol 3-phosphate dehydrogenase. Supporting this is the observation that incorporation of  $^{14}\text{C}$  labelled glycerol into ethanol insoluble material was not very much reduced in ID16D as compared to the wild-type (Figure 6.4), and any metabolism would require phosphorylation and reduction although the latter is not required for lipid synthesis.

Table 6.1

Strain	Time	
	$t_0$	$t_5$
S41	0.092	1.43
ID16D	0.067	2.60

Levels of glycerol kinase in S41 and ID16D at time zero and five hours after resuspension of logarithmically growing cells in YEPD to YEFG. Specific activity is expressed as  $\text{nanomoles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ . Samples were harvested by centrifugation, washed twice in 0.1M Tris-HCl pH 7.4 and broken by vibromixing with glass beads. The cell suspension after removal of glass beads was centrifuged at 14,000G for twenty minutes and the supernatant used in the assays.

Table 6.2

Strain	Time	
	$t_0$	$t_5$
S41	2.08	6.34
ID16D	0.53	7.68

Levels of glycerol-3-phosphate dehydrogenase in S41 and ID16D at time zero and five hours after resuspension of logarithmically growing cells in YEPD to YEFG. Specific activity is expressed as  $\text{nanomoles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ .

Derepression of markers of mitochondrial function in wild-type and sporulation derepressed strains.

Acetate, lactate, glycerol and pyruvate, that is the carbon sources on which the spd mutants do not grow, all require functional mitochondria for their utilisation as energy sources. It seems probable that functional mitochondria are also required for sporulation, as petites, which lack complete mitochondrial function, do not sporulate (Ephrussi 1953). It seems unlikely that the spd phenotype could be the result of a mitochondrial defect in energy production. However, there seems to be a lack of certain knowledge of the energy requirements of sporulation as compared to those for growth. For example if sporulation were less energy demanding it is conceivable that a reduction in mitochondrial activity could perhaps permit sporulation but not growth.

Alternatively an alteration in the control of derepression of certain enzymes involved in oxidative metabolism such that derepression was only partial or retarded may produce a situation conducive to sporulation. Two enzymes were selected for study as likely indicators of an alteration in this area of metabolism in the spd mutants.

Isocitrate lyase, as an indicator of the glyoxalate cycle, and malate dehydrogenase, as an indicator for the TCA cycle, were measured in spd1 mutant ID16D and the wild-type S41 following transfer of logarithmically growing cells from YEPD to YEP-ethanol, on which the spd mutants can grow. As seen from Figures 6.6 and 7 the derepression pattern for both enzymes was very similar in S41 and ID16D. Both strains appeared to produce the enzymes at the same rate and also reached similar levels by the

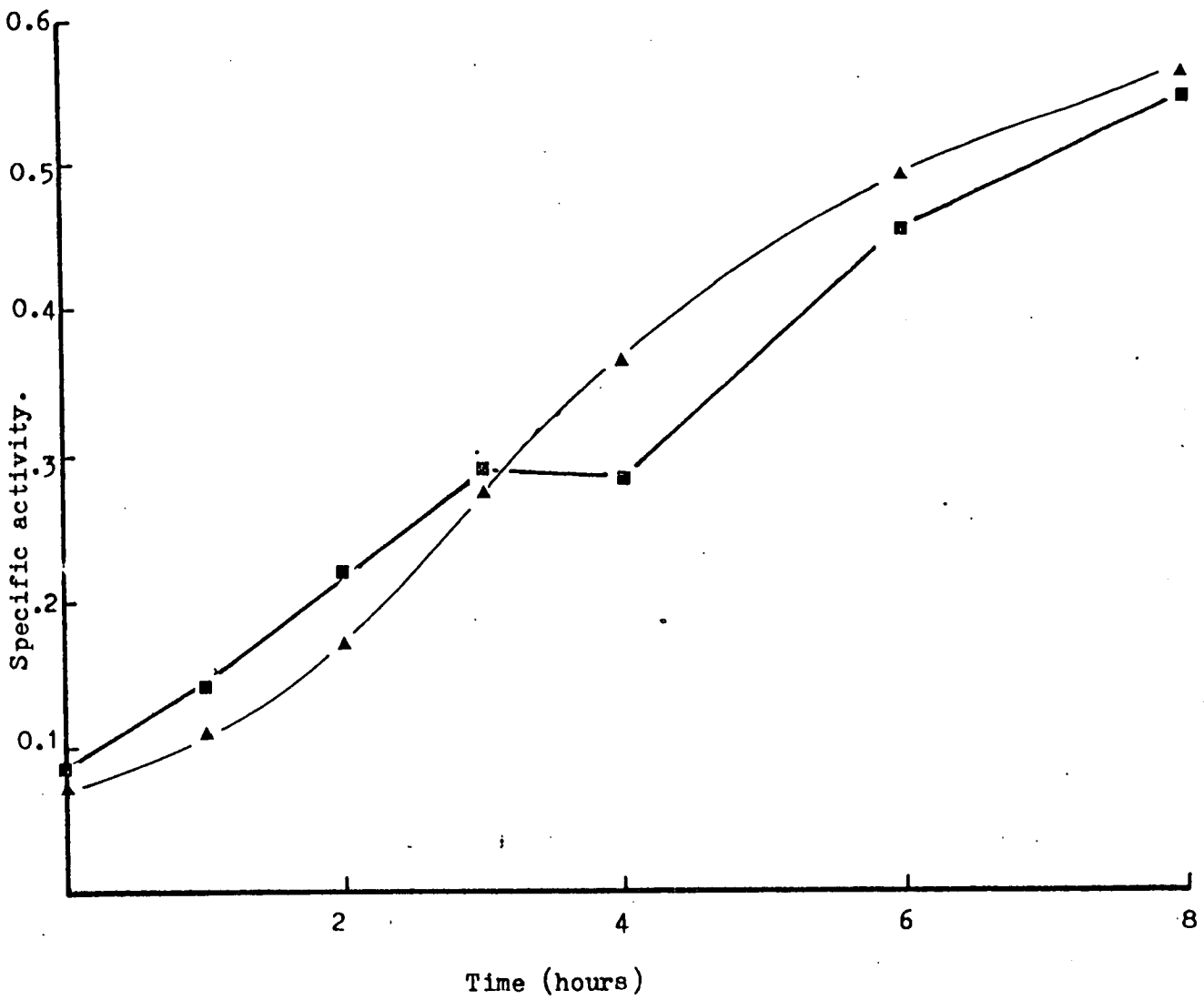
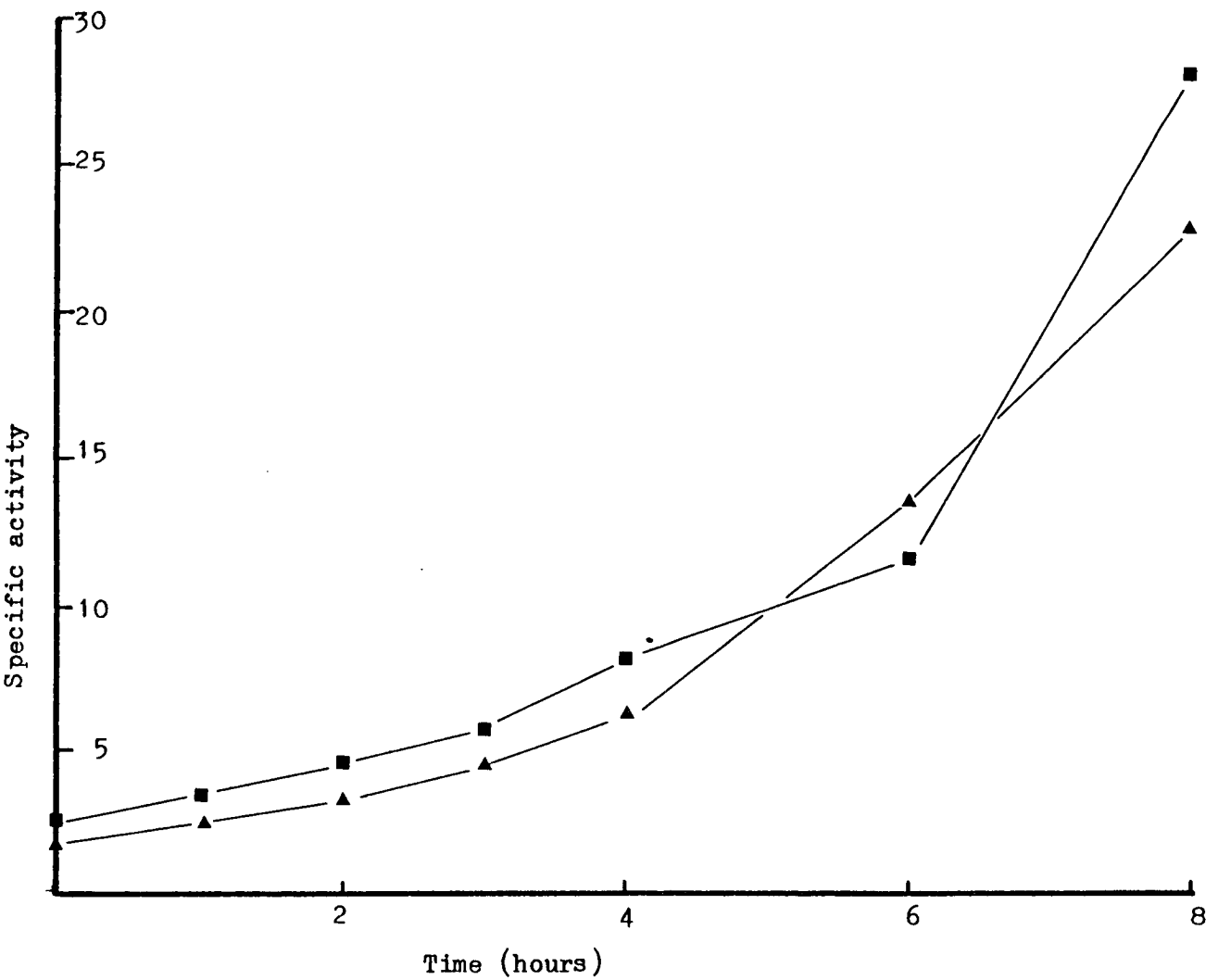


Figure 6.6

Derepression of malate dehydrogenase in S41 and ID16D after transfer of logarithmically growing cells from YEPD to YEP + 3% ethanol. Specific activities are expressed as  $\mu\text{moles NAD} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ . ■ S41; ▲ ID16D.



**Figure 6.7**

Derepression of isocitrate lyase in S41 and ID16D

following a shift of logarithmically growing cells from YEPD to YEP + 3% ethanol. Samples were harvested by centrifugation, washed with 2x20ml aliquots of 0.1M potassium phosphate pH6.5, resuspended in 5.0ml of this buffer and broken by sonication. Cell debris was removed by centrifugation and the supernatant used in the measurement of enzyme activity. Specific activity is expressed as nmol.min<sup>-1</sup> mg protein<sup>-1</sup>. ■ S41; ▲ ID16D.

end of the experiment. Initial levels, representing those of cells under conditions of repression, were also similar.

With this type of derepression experiment it was found to be important to harvest the strains from YEPD not just during exponential phase of growth but at approximately the same turbidity as it was found that later log phase cells had a tendency to exhibit higher levels, of malate dehydrogenase in particular, than earlier log phase cells. This may indicate that during growth on 2% glucose there is a gradual derepression of glucose repressible functions which occurs throughout mid-late log phase and into stationary phase. This small feature may be overcome in future experiments by using higher glucose concentrations (5%).

The derepression of the two enzymes was also followed after transfer from YEPD to YEP-glycerol as the spd phenotype is very marked on this carbon source.

Derepression of malate dehydrogenase again followed a similar pattern in wild-type and sporulation derepressed strain (Figure 6.8). The changes in turbidity during the course of the experiment are also presented (Figure 6.8) and by 24 hours there was a clear difference in turbidity between the two strains.

There was, however, found to be a considerable difference in the derepression profiles of isocitrate lyase, which was estimated at the same time as the malate dehydrogenase above. ID16D derepressed this enzyme to only approximately half the level of the wild-type during the initial stages of the experiment. There then followed a gradual decline in the levels in both strains.

By comparing the levels of isocitrate lyase reached on ethanol (23 and 27  $\text{nmoles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ) and on glycerol

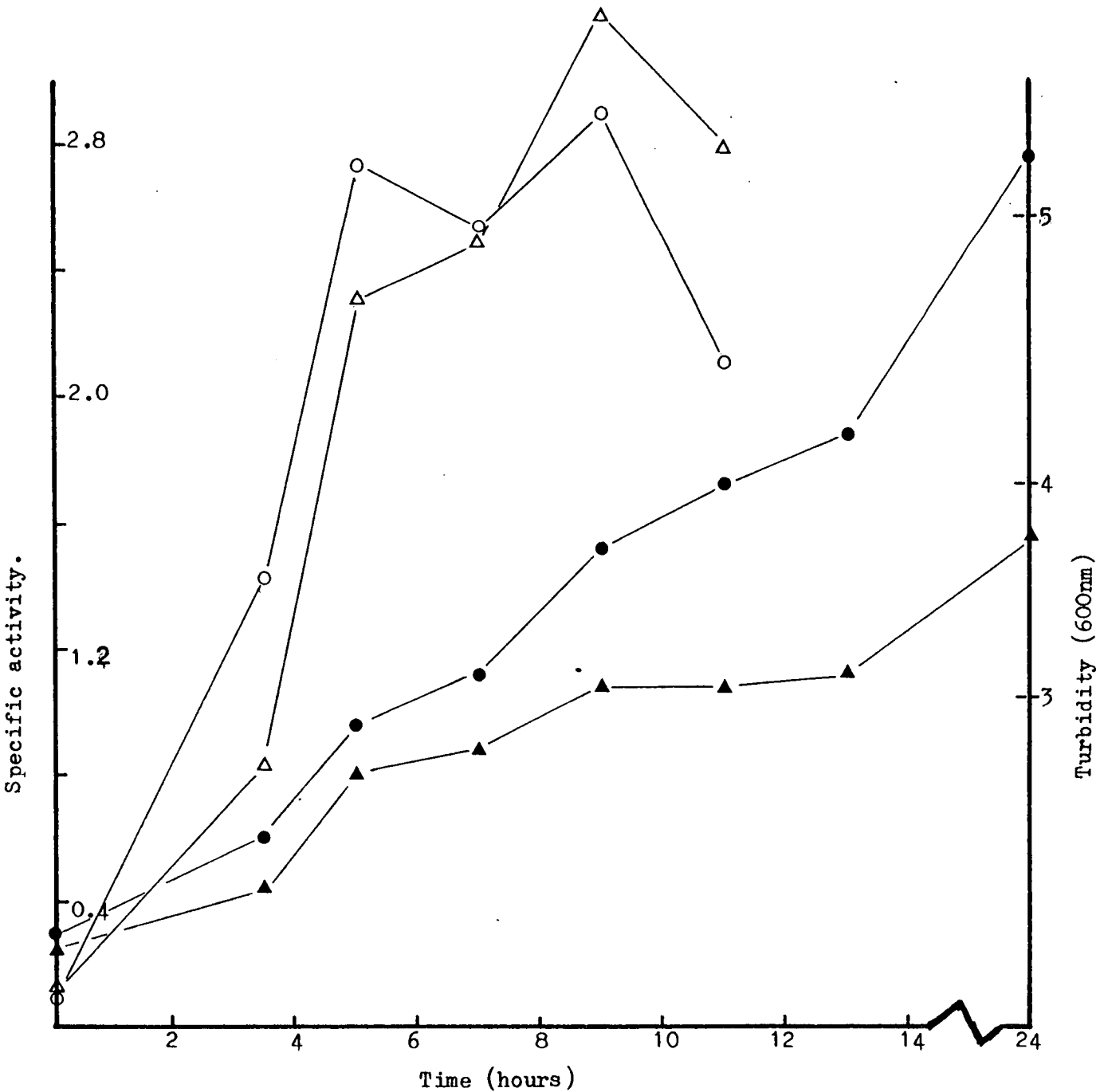


Figure 6.8

Change in the levels of malate dehydrogenase in O S41 and

Δ ID16D following a transfer of logarithmically growing cells from YEPD to YEPG. Specific activity is expressed as  $\mu\text{moles NAD}^+ \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein<sup>-1</sup>. Change in turbidity of ● S41 and ▲ ID16D in cultures after transfer to YEP-glycerol.



(9 and 14 nmoles.min<sup>-1</sup>.mg protein<sup>-1</sup>) for ID16D and S41 respectively (Figures 6.7 and 6.9) it seems that isocitrate lyase does not derepress to such a great extent on glycerol.

Therefore the only effect of the spd1 mutation on carbon metabolism is to produce rather lower levels of isocitrate<sup>lyase</sup> while adapting to YEP-glycerol medium. Whether this is a direct cause of the phenotype or merely an indication that the spd mutant had initiated sporulation during the course of the experiment (i.e. the change observed is sporulation dependent rather than growth dependent) is rather difficult to establish.

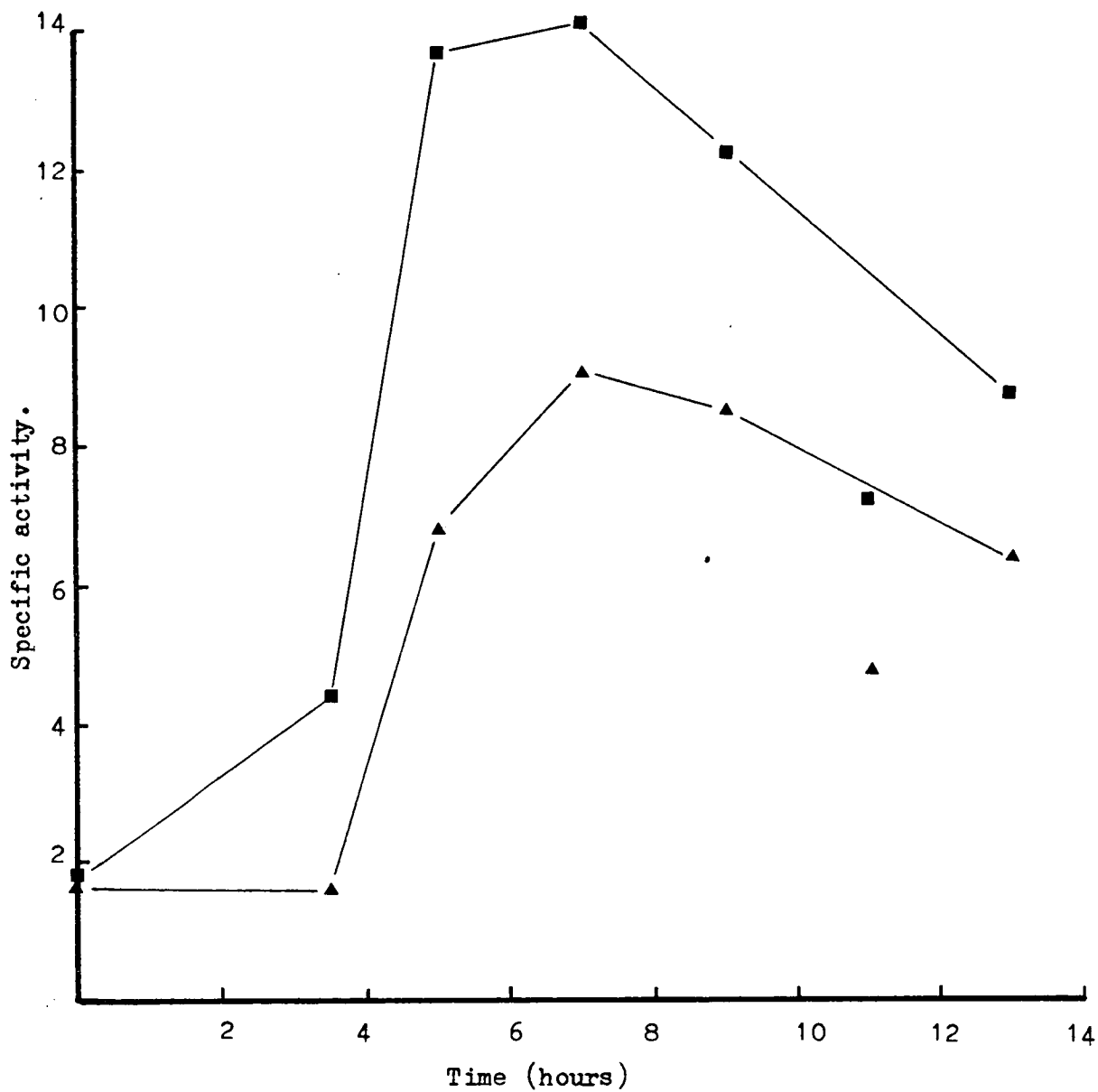


Figure 6.9

Changes in levels of isocitrate lyase in ■ S41 and ▲ ID16D following transfer of logarithmically growing cells from YEPD to YEFG.

Relationship of a strain altered in the regulation of carbon catabolite repression to the sporulation derepressed strain.

Since the 'catabolite repression' mutation (cat1-1) of Zimmermann et.al. (1977) described in the introduction, has a similar phenotype to spd1-1 mutation, with respect to the range of carbon sources affecting growth, the possibility that these two lesions were the result of a single mutation was tested.

In the course of these studies it was found that the strain CH.17-3c supplied by the authors contained three mutations affecting growth on glycerol. These three were identified from complementation tests of a dissection of strain CH.17-3c crossed to haploid strain  $\alpha$ ade5. Curiously one of these three mutations failed to complement spd1 and showed very high linkage to it, yet the cat1-1 mutation was shown not to be identical with this new spd1 mutation. This is best illustrated by examining the segregation patterns both for growth on glycerol and derepression of isocitrate lyase and malate dehydrogenase, in the haploid segregants from an ascus obtained from the cross CH.17-3c to  $\alpha$  ade5.

By complementation analysis it was found that the four spores had the following genotypes:

- 93.1A cat1-1 identified by derepression studies
- 93.1B spd1 complementation and linkage tests to spd1-1
- 93.1C cat1-1 spd1 gly<sup>-</sup> carrying a third unknown gene for poor growth on glycerol (gly<sup>-</sup>).
- 93.1D gly<sup>-</sup> third gene for poor growth on glycerol.

The patterns of enzyme derepression for these strains are shown in figures 6.10 and 6.11. The derepression patterns of malate dehydrogenase and isocitrate lyase for derivatives of spores

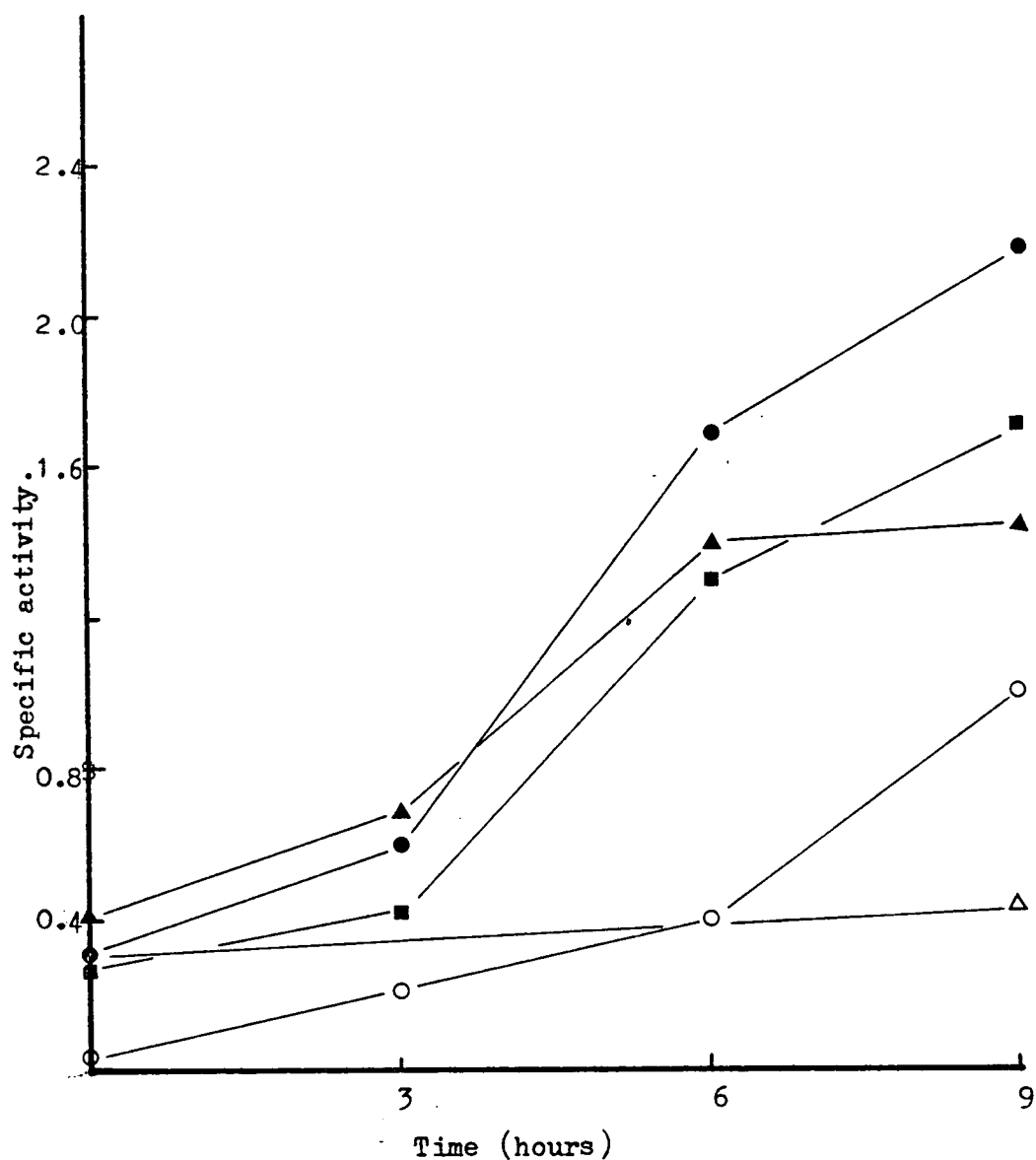
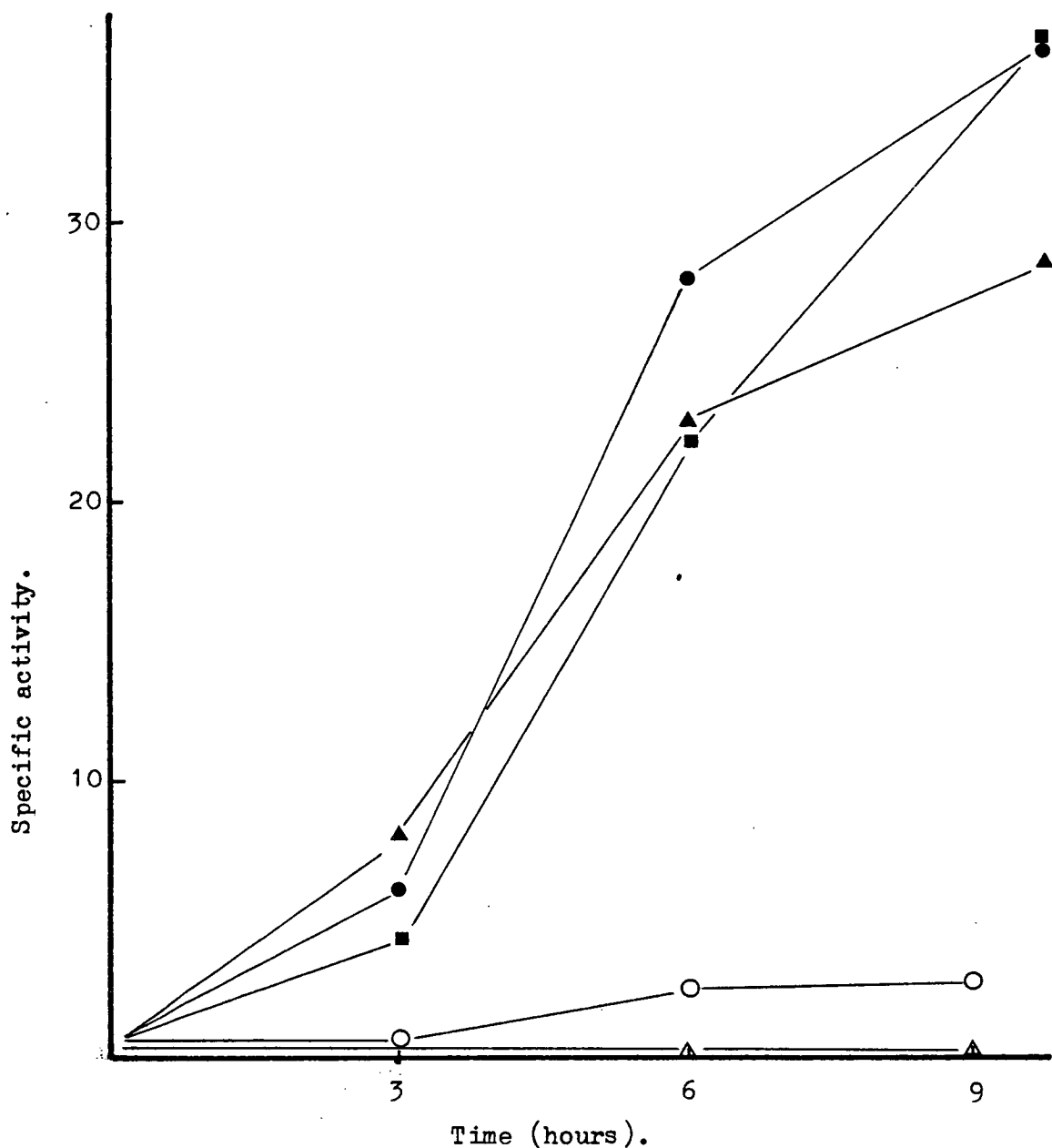


Figure 6.10

Derepression of Malate dehydrogenase in ○ 93.1A;  
 ▲ 93.1B; △ 93.1C; ● 93.1D and ■  $\alpha$  ade 5 after transfer of  
 logarithmically growing cells from YEPD to YEP with 3% ethanol.  
 These strains are the segregants of a cross of  $\alpha$  ade 5 to CH.17C  
 (*aleu1 MAL2-8<sup>c</sup> MAL3 SUC3 cat1-1*).



**Figure 6.11**

Derepression of isocitrate lyase in ○ 93.1A; ▲ 93.1B;

△ 93.1C; ● 93.1D; and ■  $\alpha$ ade 5 after transfer of logarithmically growing cells from YEPD to YEP + 3% ethanol. These strains are segregants of a cross of  $\alpha$ ade 5 to CH 17-3C (aleu 1 Mal2-8<sup>c</sup> MAL3 SUC3 cat1-1) kindly provided by F.K. Zimmermann. Specific activity is expressed as nanomoles formazan.min<sup>-1</sup>.mg protein<sup>-1</sup>.

93.1A and 93.1C coincide with the derepression profiles of strains carrying the cat1-1 mutation described by Zimmermann *et.al.*(1977). The difference between the abilities of the spd mutant and the cat1-1 strain becomes immediately obvious. The cat1-1 segregants showed almost no derepression of isocitrate lyase and delayed derepression of malate dehydrogenase, (spores 93.1A and 93.1C), and therefore:

- a). spd1 is not the same gene as cat1, nor does it affect enzyme derepression in the same way.
- b). from spore 93.1C it can be seen that the cat1 gene is epistatic to spd1 with respect to enzyme derepression.

Subsequently the cat1-1 mutation has been shown to prevent sporulation in homozygous diploids (cf. the spd1-1 phenotype leading to derepression of sporulation).

Cyclic nucleotides in wild-type and sporulation derepressed strains.

Cyclic nucleotides, in particular cyclic 3',5'-adenosine monophosphate (cAMP) are known to play important roles in the regulation of metabolism. The best understood example of the participation of cAMP is in the catabolite repression of the lactose operon. There and in other repressible operons in E.coli cAMP acts as an effector for its receptor protein (the CRP protein) to provide a positive control system for regulating enzyme synthesis (Perlman and Pastan 1971). This together with recent studies on the effects of exogenous cAMP on glucose repressible enzyme synthesis in yeast have been described in chapter 1, together with a detailed description of the evidence for a function of cAMP in the regulation of yeast sporulation. It is thus of interest to establish if the intracellular levels of cAMP in the spd mutants are altered from those of the wild-type, either during growth on glucose or after a shift to a carbon source on which the spd mutant grows poorly.

After a shift of logarithmically growing cells from YEPD to YEP-glycerol only a small overall increase in cAMP levels was observed (Figure 6.12). Both strains showed similar levels at  $t_0$  and the same trend throughout the experiment.

Changes in cAMP levels appeared to undergo extensive fluctuation (Figure 6.13). This effect has also been described by Watson and Berry (1977) during the first 12 hours of sporulation. However, there were no major differences between the two strains and certainly no evidence to suggest that the spd phenotype is caused by an under production of cAMP.

The small increases in cAMP levels on transfer to YEP-glycerol

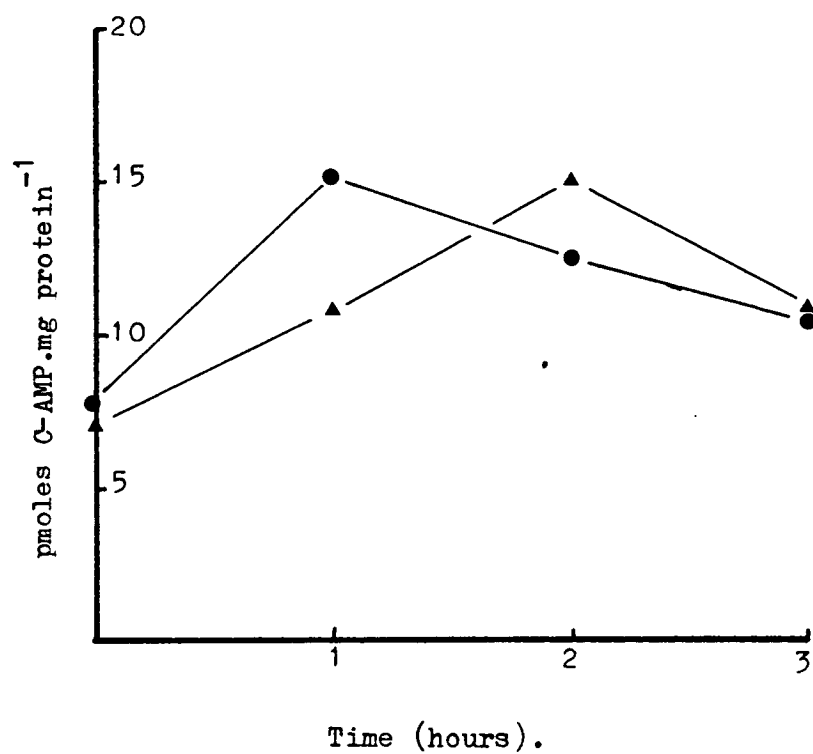


Figure 6.12

Change in levels of C-AMP following transfer of logarithmically growing cells of ● S41 and ▲ ID16D from YEPD to YEPG (a five-fold dilution). The results represent an average of two experiments.



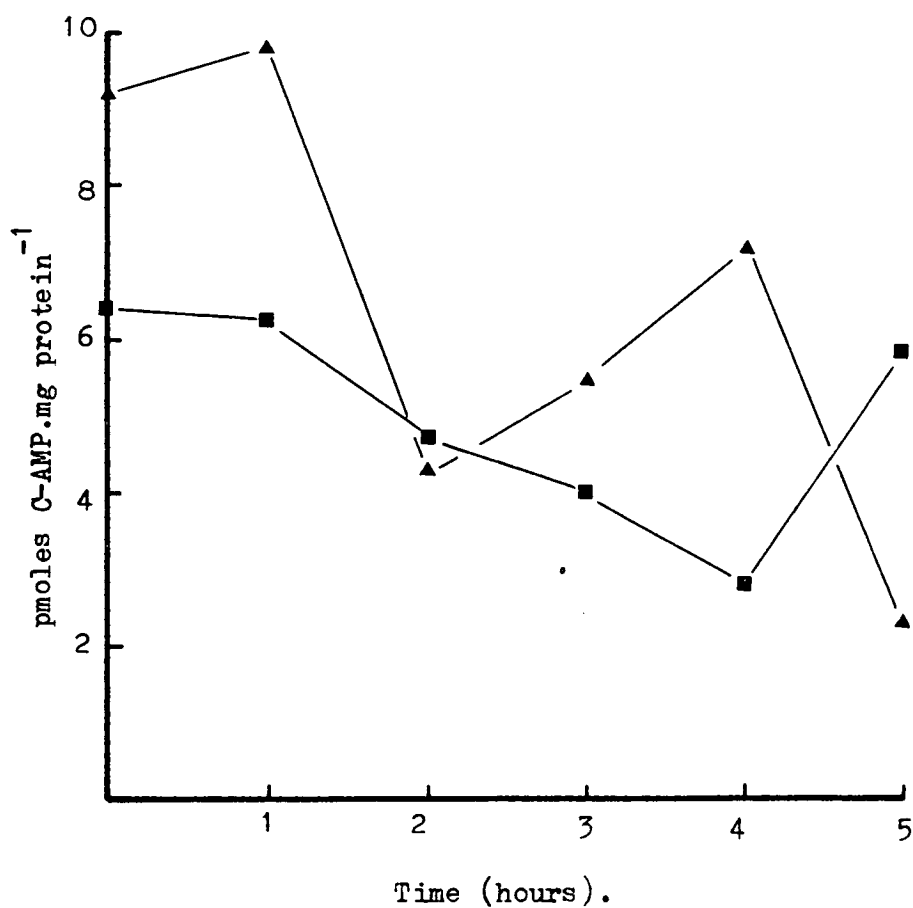


Figure 6.13

Changes in the levels of C-AMP during the early stages in sporulation. Early stationary phase glucose grown cells were transferred to 1% potassium acetate sporulation medium ■ S41;

▲ ID16D. Logarithmic cells of S41 and ID16D showed C-AMP levels of 7.7 and 8.1 pmoles.mg protein<sup>-1</sup> respectively.

medium were less than expected. Watson and Berry (1977) reported that cells grown on 2% glucose produced higher levels of cAMP than cells grown on 5% glucose. Hence this may explain the absence of a greater effect upon transfer to glycerol medium in our strains.

### Discussion.

There is no defect in the ability of the spd1 mutant to transport or incorporate acetate or glycerol, carbon sources on which it grows poorly. Neither does there appear to be any alteration in the levels of the enzymes concerned with glycerol assimilation. The only detectable difference in regulation of derepression of carbon repressible enzymes was found in the glyoxylate cycle, where the spd1 mutant ID16D failed to derepress isocitrate lyase to the same extent as the wild-type upon transfer to YEP-glycerol medium. The wild-type derepressed this enzyme to twice that of the spd1 mutant. It is difficult to determine whether this could be a causative factor in producing the spd phenotype on glycerol. This point will be considered further in the general discussion (Chapter 8) but it is important to appreciate that the abnormally low levels of isocitrate lyase may be caused by the spd1 mutant having initiated sporulation in this medium. Supporting this latter possibility is the observation that on introduction to sporulation medium the derepressed levels of isocitrate lyase undergo a steady decline from  $t_0$  throughout the course of sporulation (Betz and Weiser 1976)

The indicators of oxidative metabolism, isocitrate lyase and malate dehydrogenase, derepressed normally in both mutant and wild-type on transfer to YEP ethanol medium on which the spd1 mutants grow.

In the wild-type isocitrate lyase did not undergo as marked a derepression on glycerol as it did on ethanol. This was also observed by Gonzalez (1977). It is probable that on a complex medium with glycerol as carbon source there is not a high requirement for

the glyoxylate cycle as an anaplerotic pathway as amino acids are plentiful in YEP and metabolism of glycerol gives rise to gluconeogenic precursors directly.

Under two sets of conditions changes in cAMP levels were similar in mutant and wild type. There~~is~~ therefore no evidence that the spd phenotype could be the result of abnormal levels of cAMP or that sporulation is initiated by changes in the level of this compound in mutant and wild-type.

Genetic analysis of a mutant cat1-1, which is altered in catabolite repression, showed that the spd1 mutation is unlinked to the CAT1 gene and is unaltered in any functions identified with mutation in the CAT1 gene

## Chapter 7.

### Analysis of Revertants of Sporulation-Derepressed Mutants.

The spd1 mutants all revert fairly readily either spontaneously, usually seen as isolated colonies growing up against the poor background of the spd mutant after prolonged incubation on YEP-glycerol agar, or by mutagenesis followed by selection of survivors on YEP-glycerol medium.

A study of revertants of control mutations can provide considerable information on the mechanism of regulation. This is illustrated by the work of Zimmermann *et al* (1977) on reversion of the cat1-1 mutation affecting catabolite repression. Two classes of revertants were isolated; one, cat2-1, was an external suppressor of cat1-1 and was recessive to wild-type, the other, designated CAT1-2<sup>d</sup> was allelic to CAT1 and dominant to it. These genetic studies, together with biochemical analysis enabled a hypothesis to be proposed for the mechanism of control of carbon catabolite repression in yeast.

Before selecting revertants of spd1 mutants it is important to ensure that the spd1 chosen for study is not subject to nonsense suppression. The spd1 allele used in this study was found not to be since known suppressors did not affect the expression of the spd1 gene in suitably constructed strains, and no spd1 revertants were found to contain suppressors of appropriate suppressible auxotrophic markers (I.W. Dawes pers. comm.).

The spd1 revertants studied here have been observed to fall into only two groups. Both types showed co-reversion of the glycerol non-growth and derepressed sporulation phenotypes. One type of revertant showed normal wild-type sporulation frequencies both in

sporulation medium and after growth on glycerol. Hence it is probable that this group represented a simple reversion to wild-type.

The other much more interesting type of spd1 revertant was characterised not only by its ability to grow on glycerol but also by its failure to exhibit normal sporulation on potassium acetate medium. It is this latter type that was initially selected for study, the results of which are presented in this chapter.

From table 7.1 it can be seen that some of these non-sporulating revertants produced few recognisable asci under these conditions and none produced asci on YEP-glycerol medium, even after prolonged incubation.

It should be noted that these revertants were selected on the basis of a change in their growth phenotype and not directly for their sporulation character. This selection of mutants which can grow but not sporulate from a parent culture placed under conditions in which sporulation is more predominant than growth strongly favours selection of mutants defective in the very early stages of sporulation i.e. mutants possibly altered in initiation or at least in a sporulation event occurring before the parent strain becomes committed to sporulation (Dawes 1975; see also Piggot & Coote 1976 for discussion of a similar situation in Bacillus).

Four revertants of this type (rev 42, rev 65, rev 71 and rev 77) have been analysed genetically in some detail. Each was found to be due probably to a single nuclear mutation unlinked to the spd1 gene (by analysis of the cross to a wild-type haploid, <sup>(SH1D-5c)</sup> see table 7.2). The relative proportions of ascus types established from this cross are summarised in table 7.3. The ratio of parental

Table 7.1

Summary of spd revertant characteristics as compared to S41 and ID16D, (++ = good; +/- = poor).

Strain	YEPG growth	YEPG sporulation	KAc sporulation
S41	++	10-20%	60%
ID16D	+/-	60-70%	60%
rev 42	++	0	5%
rev 65	++	0	0
rev 77	++	0	20%
rev 23	++	0	10%

Table 7.2

rev 42	Growth		Mating type	%sporulation		Genotype	Ascus type
Ascus	YEPD	YEPG	a/ <del>α</del> /D	YEPG	KAc		
1 A	+	+/-	a	--	--	spd !+	NPD
B	++	-	D	25	30	spd +	
C	+	+	<del>α</del>	--	--	+ rev	
D	+	+	D	0	0	+ rev	
2 A	+	++	<del>α</del>	--	--	+ +	T
B	++	++	D	0	10	+ rev	
C	+	+	D	10	10	spd rev	
D	+	-	a	--	--	spd +	
4 A	+/-	-	D	--	--	spd +	T
B	++	++	D	0	0	+ rev	
C	++	+/-	<del>α</del>	--	--	+ +	
D	+	+	a	--	--	spd rev	
7 A	+	-	a	--	--	spd rev	T
B	+	+	<del>α</del>	--	--	+ +	
C	+	-	D	70	80	spd +	
D	++	++	D	0	12	+ rev	
8 A	-	-	-	--	--	+ rev	NPD
B	+	+	D	0	0	+ rev	
C	+	-	D	50	25	spd +	
D	++	-	<del>α</del>	--	--	spd +	
9 A	++		a	--	--	+ +	NPD
B	+		D	30	0	spd rev	
C	++		D	0	20	+ +	
D	+		<del>α</del>	--	--	spd rev	
10 A	+		D	0	0	+ rev	NPD
B	++	++	a	--	--	+ rev	
C	++		a	--	--	spd +	
D	+		D	30	1	spd +	
rev 65							
1 A	+	++	D	0	0	+ rev	T
B	+	++	a	--	--	+ +	
C	+	-	a	--	--	spd +	
D	+	-	D	30	30	spd rev	



Table 7.2 Key.

Growth: ++ very good; + good; +/- poor.

Mating type: D gene for homothallism giving rise to diploidy.

Genotype: + wild-type allele; spd sporulation derepressed allele; rev revertant allele.

Genotypes were assigned using three main criteria:

- a. Glycerol non-growth (spd +), glycerol growth (spd rev, ++ or + rev).
- b. Ability to sporulate extensively on glycerol medium (homozygous for spd and either homozygous wild-type or heterozygous at the reversion locus).
- c. Poor sporulation on potassium acetate (homozygous at the reversion locus).

Haploid genotypes were assigned on the basis of information obtained from a complementation matrix set up using haploids of known and unknown genotypes. In certain cases it was possible to assign genotypes on the basis of a single gene segregation

Notes.

\* assigned on the basis of a single gene segregation

▲ abnormal spores, 2-spored asci poorly formed

-- further information on haploid genotypes derived from a complementation matrix

Table 7.2 cont.

rev 65 Ascus	Growth YEPD YEPG		Mating type a/ $\alpha$ /D	% Sporulation YEPG KAc		Genotype	Ascus type
2 A	+	++	D	0	0	+ rev	NPD
B	+	++	a	--	--	spd +	
C	+	++	a	--	--	+ rev	
D	+	++	D	35	60	spd +	
3 A	+	++	$\alpha$	--	--	+ +	T
B	+	-	D	60	60	spd +	
C	+	++	a	--	--	+ rev	
D	+	++	D	0	0	spd rev	
4 A	+	-	a	--	--	spd +	T
B	+	++	D	0	0	+ rev	
C	+	++	$\alpha$	--	--	spd rev	
D	+	++	D	0	50	+ +	
5 A	+	++	$\alpha$	--	--	spd rev	T
B	+	+	a	--	--	+ rev	
C	+	+/-	D	25	20	spd +	
D	+	++	D	0	15	+ +	
6 A	+	++	a	--	--	+ +	PD
B	+	+/-	$\alpha$	--	--	spd rev	
C	+	+	D	0	0	spd rev	
D	+	++	D	0	50	+ +	
7 A	+	+/-	a	--	--	spd +	NPD
B	+	-	D	40	60	spd +	
C	+	++	D	0	0	+ rev	
D	+	++	$\alpha$	--	--	+ rev	
8 A	+	+/-	$\alpha$	--	--	spd +	T
B	+	+	D	0	0	+ rev	
C	+	+	D	1	1	spd rev	
D	+	++	?	--	--	+ +	
9 A	+	++	D	0	0	+ rev	NPD
B	+	+/-	a	--	--	spd +	
C	+	-	$\alpha$	--	--	spd +	
D	+	++	D	0	5	+ rev	
10 A	+	++	D	0	75	+ +	T
B	+	+	$\alpha$	--	--	spd rev	
C	+	++	D	0	0	+ rev	
D	+	-	a	--	--	spd +	

Table 7.2 Key.

Growth: ++ very good; + good; +/- poor.

Mating type: D gene for homothallism giving rise to diploidy.

Genotype: + wild-type allele; spd sporulation derepressed allele; rev revertant allele.

Genotypes were assigned using three main criteria:

- a. Glycerol non-growth (spd +), glycerol growth (spd rev, ++ or + rev).
- b. Ability to sporulate extensively on glycerol medium (homozygous for spd and either homozygous wild-type or heterozygous at the reversion locus).
- c. Poor sporulation on potassium acetate (homozygous at the reversion locus).

Haploid genotypes were assigned on the basis of information obtained from a complementation matrix set up using haploids of known and unknown genotypes. In certain cases it was possible to assign genotypes on the basis of a single gene segregation

Notes.

\* assigned on the basis of a single gene segregation

▲ abnormal spores, 2-spored asci poorly formed

-- further information on haploid genotypes derived from a complementation matrix

ditype asci to non-parental ditype asci clearly indicated that there was no linkage between the reversion events in rev 42 or rev 65 and the spd1-1 loci. From this cross to a wild-type haploid, haploid spores of a and α mating type were obtained carrying the spo mutation (i.e. the reversion mutation) with or without the original spd1 mutation. The crosses between these haploid strains allowed identification of the genotypes of the haploids by analysing the sporulation characteristics of the diploids formed. This also enabled complementation tests between the mutations in the four independent revertants to be carried out, as well as a complete analysis of their dominance and epistasis relationships to the spd1 mutation. Moreover, complete sets of homozygous diploids (i.e. homozygous for spo, spd1, spd1 spo and SPD SPD) were obtained from such crosses for use in future studies on sporulation.

The results of the complementation tests indicate that all four reversion mutations led to asporogeny and were recessive to wild-type. Three of the four (from strains rev 42, rev 65 and rev 77) failed to complement each other and therefore probably represent three alleles at the same locus, described as spo0-1 (from rev 42), spo0-2 (from rev 65) and spo0-3 (from rev 77). The fourth (from rev 71) was only partially asporogenous and its assignment is as yet uncertain.

The dominance and epistasis relationships of spd1-1 and spo0-1 mutations are outlined in table 7.4. Also summarised is the complementation analysis between spo0-1 and spo0-2. It can be seen that the spo0-1 mutation is epistatic with spd-1 suppressing the capacity of homozygous spd1 spo0-1 mutants to sporulate.

Table 7.3

Summary of linkage analysis data of spd1 to rev 42 & 65.

Cross	Ascus type		
	PD	NPD	T
<u>spo0-1</u> <u>spd1-1</u> <u>SP00</u> <u>SPD1</u>	1	3	3
<u>spo0-2</u> <u>spd1-1</u> <u>SP00</u> <u>SPD1</u>	1	3	6
Total	2	6	9

Summary of proportions of ascus types obtained from a dissection of crosses of sporulation derepressed strain 16D carrying two separately isolated reversions rev 42.1 and rev 65.1 to a standard haploid tester strain SHID5C.

spo0-1 (rev 42); spo0-2 (rev 65); SPD wild-type allele at spd locus; SP00 wild-type allele at spo locus.

Strain		% sporulation	
		YEPG	KAc
<u>SP00</u> <u>SPD1</u>		5	> 30
SP00 SPD1			
<u>spo0-1</u> <u>SPD1</u>		0	> 30
SP00 SPD1			
<u>spo0-1</u> <u>SPD1</u>		0	0
<u>spo0-1</u> SPD1			
<u>spo0-1</u> <u>spd1-1</u>		0	0
<u>spo0-1</u> SPD1			
<u>spo0-1</u> <u>spd1-1</u>		5	5
<u>spo0-1</u> <u>spd1-1</u>			
<u>spo0-1</u> <u>spd1-1</u>		10	> 30
SP00 SPD1			
<u>spo0-2</u> <u>SPD1</u>		5	> 30
SP00 SPD1			
<u>spo0-2</u> <u>SPD1</u>		0	0
<u>spo0-2</u> SPD1			
<u>spo0-2</u> <u>spd1-1</u>		0	5
<u>spo0-2</u> SPD1			
<u>spo0-2</u> <u>spd1-1</u>		5	5
<u>spo0-2</u> <u>spd1-1</u>			
<u>spo0-2</u> <u>spd1-1</u>		> 30	> 30
SP00 <u>spd1-1</u>			
<u>spo0-2</u> <u>spd1-1</u>		5	5
<u>spo0-1</u> <u>spd1-1</u>			
<u>spo0-2</u> <u>SPD1</u>		0	5
<u>spo0-1</u> SPD1			

Table 7.4

Summary of dominance and epistasis relationships of rev 42 and rev 65 to wild-type and spd1-1 strains derived from analysis of sporulation behaviour of progeny from crosses of appropriate haploid strains.

SPD1 is wild-type, spd1-1 derived from ID16D, spo0-1 from rev 42 and spo0-2 from rev 65. SP00 is wild-type at the ~~reversion~~ locus.

In those revertants which do sporulate slightly in potassium acetate, asci began to appear at the same time as in the wild-type S41 (Figure 7.1) but the overall numbers of asci formed was much reduced and they accumulated at a slower rate.

During logarithmic growth on YEP-glucose or YEP-glycerol these non- or poor-sporulating spo0 revertants had a normal 'budding' yeast appearance. However, stationary phase cultures showed a high tendency to become very pseudomycelial in appearance forming long chains of cells. A high number of aberrantly budded cells was also found in stationary cultures. Wild-type S41 does not become pseudomycelial after the end of growth on YEP-glucose and does sporulate to a small extent after prolonged incubation. The viability of spd1 revertants was observed to be greatly reduced after prolonged incubation on YEP-glucose (approximately two weeks on solid medium), whereas wild-type strains retain viability for considerable periods under these conditions.

Glycogen accumulation after replacement in sporulation medium in wild-type and spd1 revertants.

Mutants have been reported which fail to accumulate glycogen (Rothman-Denes and Cabib 1970). These have been examined physiologically and genetically by Pringle (1972) who established that the inability to accumulate glycogen was due to a recessive mutation, which also caused several physiological effects:

1. a much higher than normal frequency of budded cells was observed in stationary phase cultures of mutant strains, which suggested to the authors that the normal transition from growth stage to stationary phase was disrupted.
2. the viability of mutant strains declined much more rapidly than in wild-type strains during extended incubation in stationary

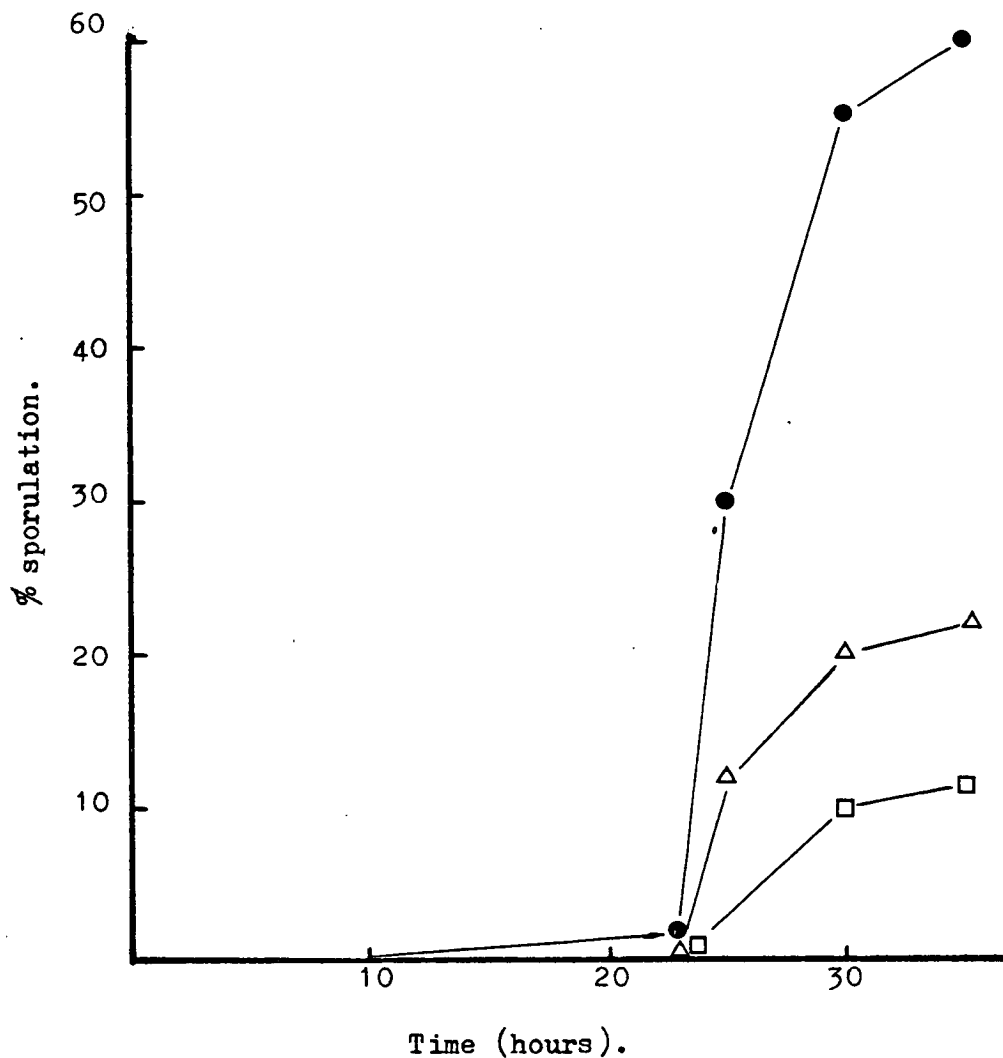


Figure 7.1

Sporulation kinetics of ● S41; and mutants Δ rev77 and □ rev23. Strains were pre-grown on YEPA overnight then used as an inoculum for YEPA. Logarithmically growing cells in YEPA were transferred with one wash in distilled water to 1% potassium acetate sporulation medium. Sporulation frequencies were determined as described in chapter two.



phase.

3. diploids homozygous for the mutation showed much poorer sporulation than did heterozygotes or homozygous wild-type strains.

Since these traits are very similar to those exhibited by the spo0 revertants of the spd1 homozygous diploids, the abilities of these revertants to accumulate glycogen under two different conditions was examined.

Initially the ability of the revertant strains to accumulate glycogen was tested after three to four days growth on YEPD plates, i.e. at the onset of stationary phase by the iodine staining method of Chester (1967). Colonies which have accumulated glycogen turn a characteristic brown colour when the plate is flooded with a solution of iodine. All revertant colonies tested showed this colour change. In contrast, petite mutants which appear spontaneously at a low frequency in yeast and are characterised by their small size and pale colour, which were observed occasionally on these plates amongst the spd revertant colonies gave a yellowish-white colour as a result of failure to accumulate glycogen.

Further, glycogen accumulation following resuspension of logarithmic YEP-acetate grown cells of S41 and rev 77 (spo0-3) into sporulation medium was examined. rev 77 sporulates very poorly, but the glycogen accumulation pattern in both strains was almost identical (Figure 7.2). Therefore, no defect in ability to manufacture glycogen was found in the revertants which could account for their asporogeny. Glycogen accumulation is an early event in sporulation and, as the revertants are obviously blocked early in sporulation, either glycogen accumulation occurs prior to the spo0 block or more probably, initiation of the sporulation programme per se is not essential to glycogen accumulation although

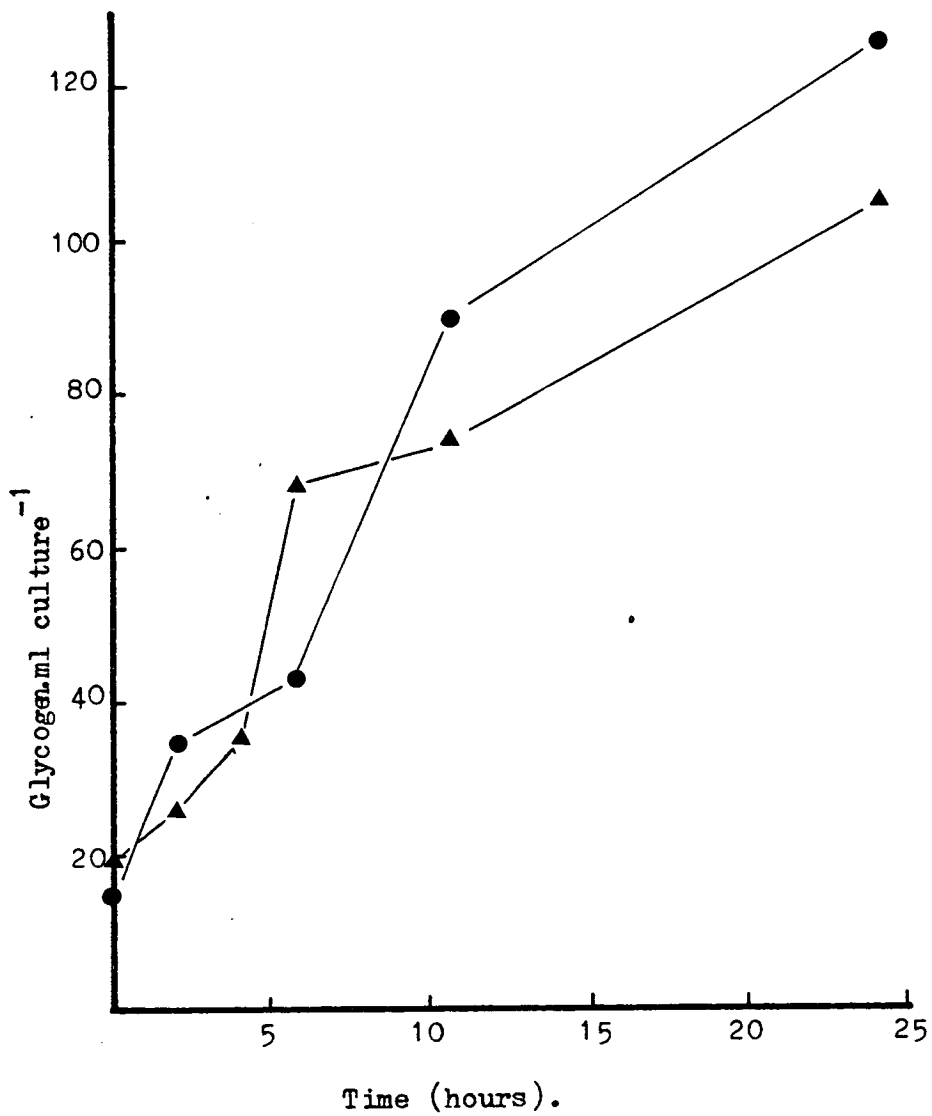


Figure 7.2

Glycogen accumulation during sporulation in ● S41; and ▲ rev77. Glycogen is expressed as  $\mu\text{g glucose.ml}^{-1}$  in the culture. Cultures were resuspended in potassium acetate sporulation medium from YEP-acetate to a similar turbidity.

the latter is necessary (from the results of Pringle 1972) to sporulation. The appearance of revertant strains of spd1 mutants following starvation tends to support the latter alternative.

Derepression of carbon catabolite repressible enzymes in spd revertants rev 42 and rev 65.

A return to the ability to grow on glycerol is one of the main features of the spd1 revertants. Although there has been no alteration found in carbon metabolism in an spd1 mutant apart from the 50% reduction in the ammount of isocitrate lyase produced on transfer from YEPD medium into YEP-glycerol, it does not rule out the possibility that a reversion to growth on glycerol and acetate, particularly if it affects a protein involved in regulation could also give rise to an alteration in some aspects of carbon metabolism. The revertants of the cat1-1 mutation, for example, show earlier derepression of certain enzymes (Zimmermann et.al. 1977).

Derepression of malate dehydrogenase and isocitrate lyase following transfer to YEP-ethanol medium was similar in S41, ID16D, rev42 and rev65 (Figures 7.3 and 7.4). Glucose repressed levels were the same in all strains.

The levels of glycerol-3-phosphate dehydrogenase and glycerol kinase were measured in rev 42 and rev 65. Again, basal levels and those at five hours after resuspension in glycerol-containing medium were similar in all strains (Table 7.5).

Discussion.

To date therefore no biochemical alteration has been established in any of the revertants tested. However, some of the morphological and physiological observations may eventually assist in determining the nature of the reversion. Certainly the pseudomycelial 'budded' appearance of stationary phase cells reflects an imbalance or

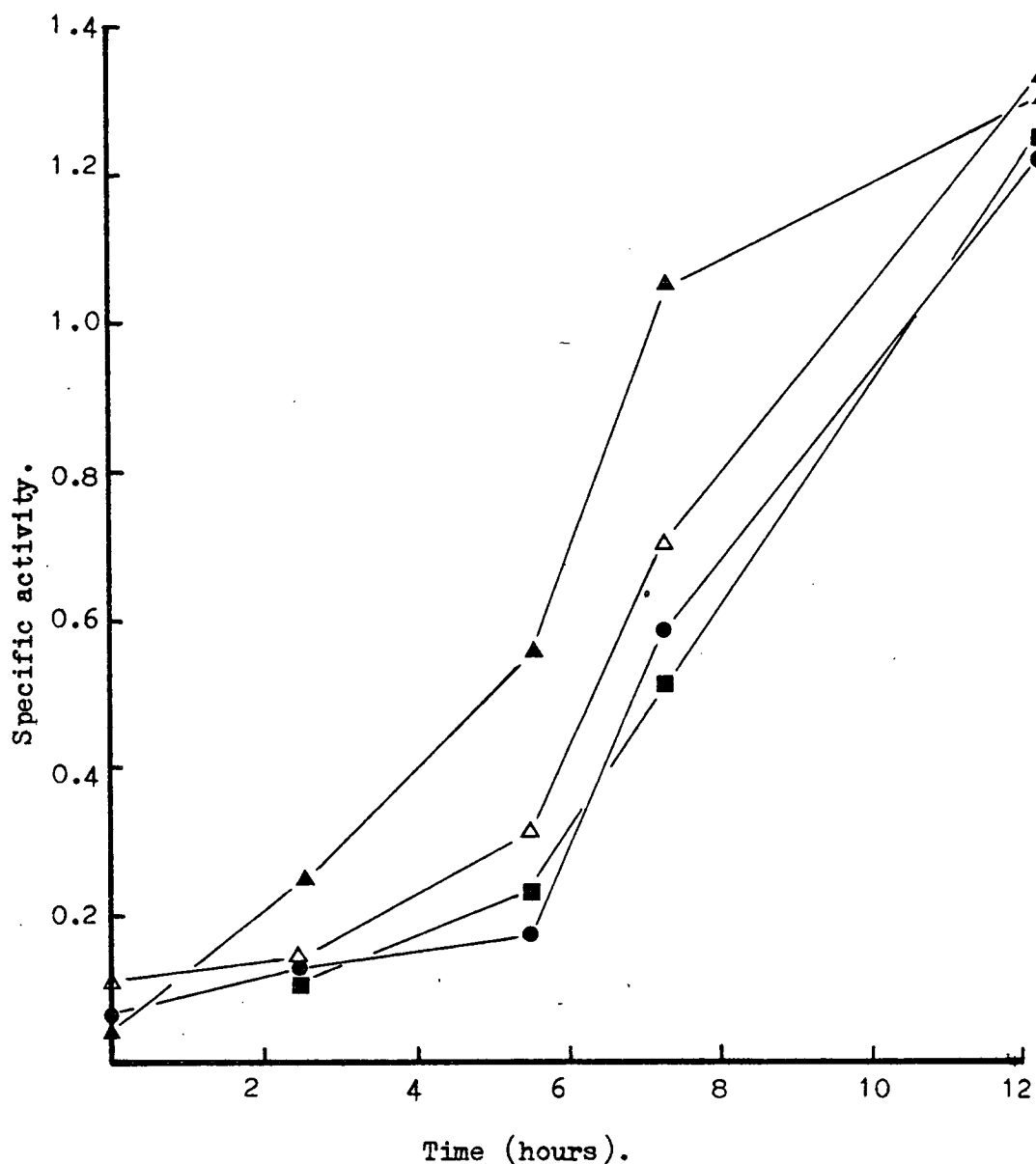


Figure 7.3

Derepression of malate dehydrogenase in  $\Delta$  S41;  $\blacktriangle$  ID16D; rev1;  $\blacksquare$  rev2.2; following transfer of logarithmically growing cells from YEFD to YEP with 3% ethanol. Specific activities are expressed as  $\mu\text{moles NAD}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ .

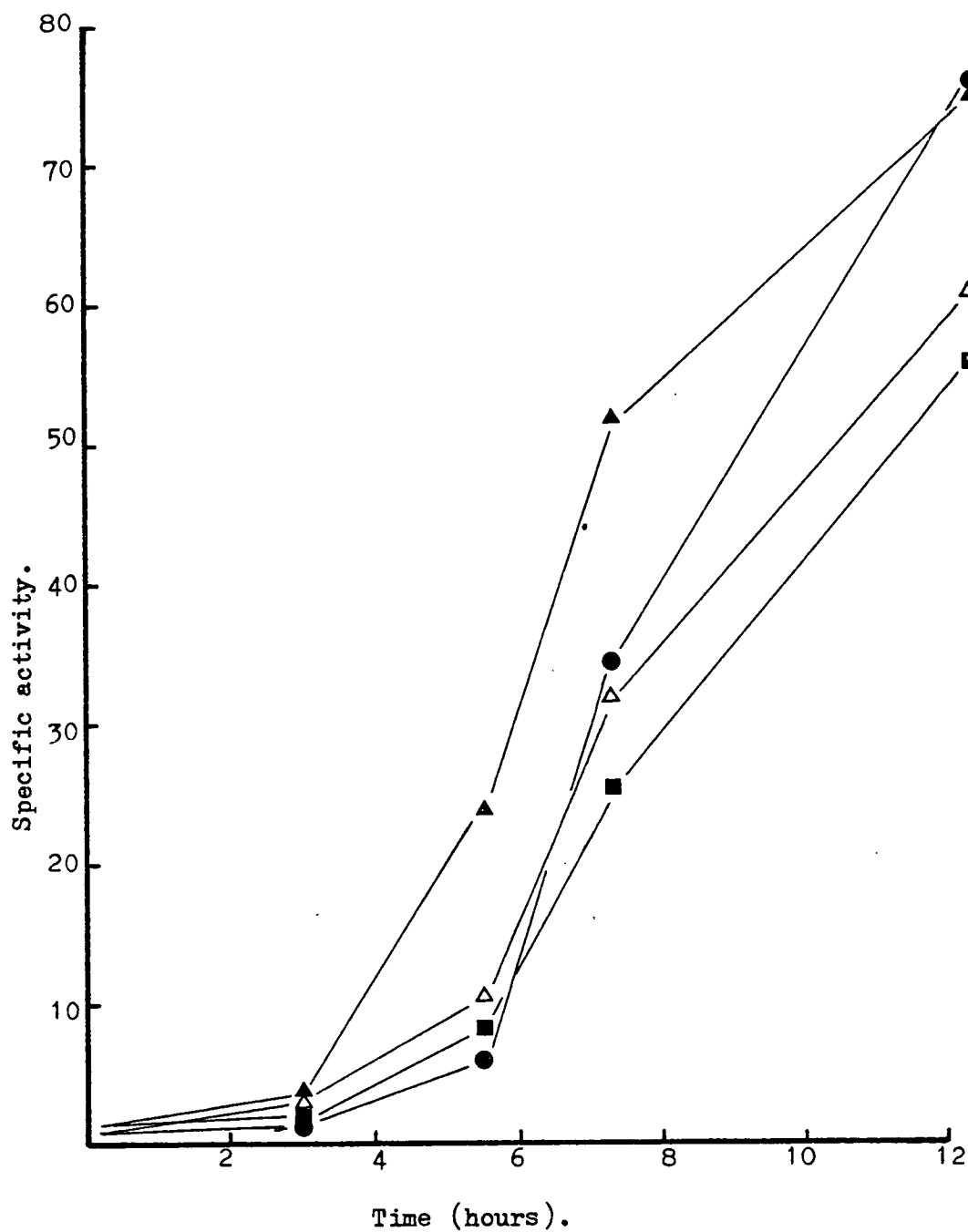


Figure 7.4

Derepression of isocitrate lyase in  $\Delta$  S41;  $\blacktriangle$  ID16D;

$\bullet$  rev1;  $\blacksquare$  rev2; following transfer of logarithmically growing cells from YEPD to YEP with 3% ethanol. Specific activities are expressed as nanomoles formazan.min<sup>-1</sup>.mg protein<sup>-1</sup>.

Table 7.5

Levels of glycerol-3-phosphate dehydrogenase and glycerol kinase in S41, ID16D and revertants 1 and 2.2 at  $t_0$  and  $t_5$  five hours after resuspension of cells growing logarithmically in YEPD to YEPG.

Strain	$t_0$	$t_5$
Glycerol 3-phosphate dehydrogenase		
S41	2.08	6.34
rev1	0.40	5.15
rev2.2	0.32	7.78
ID16D	0.53	7.68
Glycerol kinase		
S41	0.092	1.43
rev1	0.040	1.85
rev2.2	0.026	1.76
ID16D	0.067	2.60

or perhaps insensitivity of the control of the cell growth and division cycle in its response to starvation conditions. The likelihood that in many cell types division and cell development are mutually exclusive may be reflected in the failure of the revertants to sporulate since they appear altered in the 'starvation' control of cell growth and division.

Of course it cannot be ruled out at the moment that the non-sporulating revertants are blocked at some point downstream from initiation. There are few early sporulation-specific marker events in yeast (c.f. Bacillus subtilis , Mandelstam 1969). Further work to test this possibility might include testing some of the revertants for the ability to undergo meiotic recombination which is specific to sporulation (Esposito and Esposito 1974) by building appropriate tester strains carrying the spo0 suppressor of spd1-1.

Phenotypically the spo0 containing revertants of spd1 strains are extremely interesting and unusual and it seems that further research into the physiology and biochemistry of these strains must provide further information on the mechanism of control of sporulation.

## Chapter 8.

### DISCUSSION AND CONCLUSIONS.

In the results section various hypotheses as to the nature of the spd1 lesion were proposed and examined. These included:-

1. An alteration in the nitrogen control of sporulation.
2. A defect in carbon metabolism leading to starvation and "derepressed" sporulation.
3. An alteration in the cell cycle control of growth, division and development.

Experimental study of the spd1 mutants also provided considerable new information on the physiological circumstances surrounding sporulation in the wild-type. In this discussion these hypotheses will be considered in greater depth and extended to provide possible models for the regulation of sporulation.

#### Nitrogen control of sporulation.

It was originally proposed that the spd1 phenotype defined by Dawes (1975) for YEP-glycerol medium resulted from a lesion specifically involved in the nitrogen control of sporulation, i.e. the nitrogen control had been lost as sporulation but not growth occurred in the presence of a rich nitrogen source. There is ample evidence in the literature (see chapter 1 for refs.) to support the existence of both a carbon and a nitrogen control which must be released to allow sporulation to occur in yeast. In this thesis the failure of our wild-type to sporulate on YEP-acetate medium illustrates the repressive effect of a nitrogen source.

These original observations on the spd1 mutants have been extended in this work to include other non-fermentable carbon



sources; pyruvate, lactate, succinate, acetate. However, the spd1 phenotype was not observed on media containing galactose or ethanol as energy source. The ability of the mutants to grow on galactose is perhaps not surprising as although it does not repress the TCA cycle enzymes as efficiently as glucose it does repress the glyoxylate cycle (Polakis and Bartley 1965). Therefore cells do not become fully derepressed for carbon-repressible functions on galactose. Ethanol, on the other hand has been found in this study, and elsewhere (Gonzalez 1977), to cause an extensive, possibly maximal derepression of TCA and glyoxylate cycle enzymes. This derepression occurred to an equal extent in spd1 mutants and wild-type. Therefore in this situation where the spd1 mutants are fully derepressed for carbon catabolite repressible functions yet maintain growth in the presence of a nitrogen source, it is unlikely that they are altered in the nitrogen control of sporulation.

Dawes (1975) however showed that sporulation in the spd1-1 mutant ID16D was insensitive to the ammonium ion in the presence of low concentrations of glucose, whereas the wild-type remained sensitive. Sporulation in the wild-type is extremely sensitive to inhibition by low concentrations (0-2mM) of ammonium sulphate in acetate sporulation medium but although spd1-1 mutant ID16D appeared to have reduced sensitivity compared to the wild-type this was not so evident for the other two alleles tested. It was however noted that ammonium at high concentrations has an extremely toxic effect in the presence of acetate. Therefore it is possible that the effect of the spd1 lesion may be masked in this situation. But, as sporulation in the wild-type at low concentrations appears only to be retarded (occurring over forty-eight rather than the normal

twenty-four hours) this is unlikely. It appears that the rate of initiation of sporulation is reduced in the presence of methylamine as it is in low concentrations of ammonium sulphate. It must be mentioned however that tetrads formed in the presence of methylamine had an atypical appearance, thus this analogue could be exerting effects which ammonium does not. Pinõn (1977) has observed similar effects with ammonium ion and methylamine. Interestingly, ammonium, albeit tested at rather high concentrations (4 and 5mM) does not act to repress sporulation right at initiation but first, on pre-meiotic DNA synthesis and then later in the sequence (Durieu-Trautmann and Delavier-Klutchko 1977; Pinõn 1977). The spd1 mutants because of their ability to initiate sporulation under conditions where the wild-type does not are likely to be altered at least in the regulatory process of the primary initiating event or in the metabolism leading up to sporulation. This, together with the variable response of the spd1 mutants to the presence of a nitrogen source (i.e. sensitive under some conditions insensitive under others) strongly disfavours an alteration in nitrogen repression of sporulation as the primary cause of the spd1 phenotype. It is much more probable that the lesion lies in an area of the metabolism or regulation which can alter the ability of cells to initiate sporulation and which under certain environmental conditions, but not all, has a secondary effect on the nitrogen repression of sporulation; for example starvation for an energy source could have a deleterious effect on nitrogen source uptake or metabolism. The possibility that an alteration in the carbon and energy source metabolism could give rise to an spd phenotype is considered in the next section.

Intermediary metabolism in wild-type and sporulation derepressed strains.

One of the most logical conclusions on the spd phenotype is that mutants are unable to fully utilise non-fermentable carbon sources, leading to starvation and secondary effects on nitrogen source uptake and incorporation, and hence to sporulation. The spd1 phenotype is not due to a deficiency in transport and incorporation of acetate or glycerol or in the early metabolism of glycerol. This, together with the highly pleiotropic nature of the lesion indicates that the defect must lie in intermediary metabolism either in its regulation or in the activity of a key enzyme. It is important to note that this lesion cannot be one which completely blocks metabolism of a particular carbon source as sporulation cannot occur without one, nor can it prevent utilisation of the non-fermentable carbon source ethanol on which the spd1 mutants grow well. It is hard to reconcile the latter characteristic with an alteration in intermediary metabolism, but there do seem to be major differences between the properties of ethanol and acetate for example which will be considered in a later section. It is also known that certain types of mutations in intermediary metabolism do not permit sporulation to occur e.g. the respiratory deficient "petite" mutants of yeast. An alteration in the intermediary metabolism in the spd1 mutants is most likely to be in energy metabolism since in yeast extract-peptone-glycerol medium for example, the glycerol probably functions mainly as an energy source as amino acids are in plentiful supply.

Croes (1967) has in fact proposed that sporulation, under normal circumstances, might be triggered by an insufficiency of the glyoxylate cycle. This functions as an anaplerotic pathway for the

maintainence of levels of the TCA cycle intermediates for energy production when these are being siphoned off for synthesis of amino acids and other compounds. Croes observed an acceleration in metabolism on transfer to sporulation medium which the glyoxylate cycle might be unable to support. He proposed that an inadequate glyoxylate cycle activity could lead to initiation of some of the events necessary to sporulation such as protein breakdown. This theory does not provide a mechanism of the primary control events of initiation but does reflect on the possible metabolism leading to sporulation.

Interestingly the only major defect found in the spd1 mutant ID16D was an inability to undergo full derepression of isocitrate lyase in YEP-glycerol medium to the extent found in the wild-type. Although there is probably only a low requirement for activity of the glyoxylate cycle on YEP-glycerol medium, it is conceivable that during the period immediatly following transfer to glycerol medium, due to a delay in adaptation there is insufficient energy available for transport of exogenous amino acids. During the early stages of sporulation therefore there may be a demand for the operation of the glyoxylate cycle to ensure an adequate supply of amino acid precursors. A failure of the glyoxylate cycle at this critical time may be sufficient to trigger sporulation in the spd1 mutants.

The accumulation of evidence does however tend to argue against this being the area of metabolism involved:

1. There is no insufficiency of the glyoxylate cycle, at least for isocitrate lyase activity during growth on ethanol in the spd1 mutants.
2. It is doubtful that such a metabolic situation in the presence

of an adequate supply of nitrogen could support both the initiation and the maintenance of the sporulation sequence; a degree of reversibility of sporulation once metabolism had adapted to growth on glycerol could be expected for the spd1 mutants.

3. It is extremely probable that the low levels of isocitrate lyase observed in the spd1 mutant reflect only the certainty that sporulation is in progress in this YEP-glycerol medium. Betz<sup>and Weiser</sup> (1976) observed that isocitrate lyase decreased steadily from  $t_0$  in sporulation medium, suggesting that this enzyme is not required. Therefore it is more than likely that the low derepression observed represents the "struggle" between adaptation to growth on glycerol and initiation of sporulation. Again, the difficulty in distinguishing causative factors from the consequences of sporulation is evident (c.f. Figure 1.3 where the increase in levels of NADP<sup>+</sup> dependent glutamate dehydrogenase at the end of the time course in the wild-type probably only reflects adaptation to growth on glycerol in this case and sporulation in the spd1 mutant).

4. The failure of haploids to grow on YEP-glycerol is also detrimental to this hypothesis. One would expect low levels of isocitrate lyase to permit slow growth at least, perhaps following a lag. However the inability of spd1 haploid mutants to grow on these non-fermentable carbon sources does strongly indicate that some area of metabolism is altered, rather than an initiation event.

In considering intermediary metabolism the spd1 reversion event provides some useful information. The reversion site is unlinked to the spd1 locus. Therefore, unless the reversion was a suppressor of the spd1 mutation, the latter cannot be in a structural gene altering the activity of a key enzyme. The spd1 mutation is not

however subject to nonsense suppression and it must therefore be concluded that the lesion lies in a regulatory element of central metabolism. Disappointingly, in the spo O strains, reversion to growth on glycerol was not paralleled by an alteration in the repression characteristics of any of the carbon catabolite repressible enzymes studied. An alteration in carbon control in the revertants would have provided positive evidence for a defect in control of some area of intermediary metabolism in the spd1 mutants.

As mentioned previously it is difficult to reconcile growth of the spd1 mutant on YEP-ethanol medium being almost as good as was found for the wild-type, as derepression experiments showed that both strains became fully derepressed for both malate dehydrogenase and isocitrate lyase. Under these growth conditions one would expect the spd1 strains to exhibit derepressed sporulation. One possibility is that ethanol utilisation may depend on a different regulatory circuit from that controlling other non-fermentable carbon sources, however, as much of the metabolism must be common this is rather unlikely. Alternatively ethanol may inhibit sporulation and the spd1 mutants grow because they cannot sporulate but this is disputed by spd1 haploids which show the same growth pattern as spd1 homozygous diploids.

Of much more significance in the examination of this contradiction is the ability of ethanol to support good growth of the wild-type as well. As can be seen from the results of Chapter 3, growth on YEP-ethanol is considerably better than growth on YEP-acetate. This is surprising if as is reported in yeast ethanol is metabolised via acetate and thence through the TCA cycle (Sols et.al. 1971). Metabolism proceeds from ethanol via acetaldehyde to acetyl-CoA.

These results show that there is a fundamental difference between the utilisation pathways of ethanol and acetate and in fact, the spd1 mutants serve to emphasise the difference between ethanol and acetate as energy sources. A similar difference in the growth supporting properties of ethanol as compared to acetate was observed by Gonzalez (1977). The work of Polakis et.al. (1964) indicated that ethanol is capable of producing the highest respiration rates of all substrates tested in the order ethanol > glucose > acetate. Ethanol in this study was found not to support such efficient sporulation as acetate.

One possible cause of the difference in growth supporting properties is that ethanol may be able to enter the cells more readily than other such carbon sources. The relatively poor growth of our wild type on glycerol, acetate, pyruvate and succinate may indicate that entry of these compounds is slow. Polakis and Bartley (1965) state that ethanol enters yeast cells much more readily than does pyruvate. Therefore, in spite of an alteration in metabolism in the spd1 mutants, sufficient ethanol may enter the cells to permit growth.

Interestingly, neither glycerol nor pyruvate ( both C3 compounds ) functioned well as carbon sources for sporulation in wild type and spd1 mutants. However the spd1 phenotype was expressed on these carbon sources in the presence of yeast extract-peptone. This suggests that yeast extract-peptone is providing a component essential to sporulation which cannot be synthesised on pyruvate or glycerol when present alone as carbon source for sporulation. This reflects some area of metabolism which is similar in both strains. It may be that the anaplerotic function of pyruvate

carboxylase is not present in our strains.

It must be emphasised that in spite of all the biochemical observations which have been made to date, no concrete difference in the metabolism of spd1 mutants has been found, but a defect in some area of intermediary metabolism which mimics starvation when glycerol etc. are present as energy sources remains an extremely possible candidate. This is most likely to affect the regulation of enzyme levels leading to an imbalance in metabolism. If this unknown metabolic alteration is present it is complicated and pleiotropic in its effects, as starvation does not normally lead automatically to sporulation only to an accumulation in the G<sub>1</sub> period of the cell cycle. Identification of this biochemical damage is not likely to be easy but should provide considerable insight into the area of metabolism directly influencing the repression of sporulation. Areas that should be analysed include ATP production, the TCA cycle and an analysis of mitochondrial proteins in wild-type and mutant strains under different growth conditions. It may also be informative to analyse uptake of nitrogen sources during growth on different carbon sources in both strains, as it seems possible that uptake of nitrogen source could be reduced in the spd1 mutants in the presence of certain non-fermentable carbon sources. Perhaps indicative of this is an observation made recently that the spd1 mutant ID16D retained greater viability in the presence of the toxic ammonium ion / acetate combination than the wild-type suggesting possibly a certain degree of exclusion of the ammonium ion.



### Cell cycle control of sporulation

The spd1 mutants appear to exhibit the derepressed phenotype only with those carbon sources on which the wild-type also grows slowly and not on those carbon sources on which the wild type grows well (i.e. galactose and ethanol). The fact that the mutants grow well on ethanol containing medium may indicate that there is no defect in intermediary metabolism such as the TCA cycle or glyoxylate cycle. Therefore under conditions in which only a relatively slow growth rate is possible the spd1 mutants initiate sporulation at a high frequency. This implies that the spd1 mutants are altered in some aspect of cell cycle control that monitors growth rate or the transition from mitosis to meiosis. This possibility will be considered further in this section in relation to a cell cycle control on the initiation of sporulation.

The  $G_1$  period of the cell cycle is taken to be the time between cell separation and the initiation of DNA synthesis (Figure 4.5 ). During this time the cell exists in a single, unbudded state. Many forms of nutritional deprivation lead to an accumulation of unbudded cells in the  $G_1$  phase of the cell cycle ( $G_1$  arrest, Hartwell 1974). These include reaching stationary phase in rich medium, nitrogen, sulphur, biotin and phosphate starvation, but not starvation for essential auxotrophic requirements. The likelihood that yeast cells enter the pathway of development <sup>from  $G_1$</sup>  has been discussed in the Introduction (Chapter 1). This seems to be true for most cell types including mammalian cells (Vendrely and Vendrely 1956).

In this part of the discussion it is proposed that there is some form of nutritional sensor or growth rate sensor in the  $G_1$

phase of the cell cycle which is altered in the spd1 mutants and functions to direct them into the sporulation sequence rather than the growth and division cycle. It therefore follows that the spd1 reversion event in some way does not permit entry into the development pathway, instead, failure to monitor starvation conditions leads to continuation of an abnormal and abortive cell cycle.

spd1 homozygous diploids and spd1 haploids were recently found to accumulate as unbudded cells following transfer to glycerol medium. Both strains were observed to complete the cell cycle in progress at the time of the shift and sometimes initiation and completion of one more cell cycle (Vezhinet et.al. 1979). Therefore, spd1 homozygous diploids accumulate in the G<sub>1</sub> phase of the cell cycle and enter the pathway of development under conditions which normally support growth and division in the wild-type. This therefore is analagous to the behaviour of certain conditional cell cycle mutants which undergo G<sub>1</sub> arrest at the restrictive temperature. In this case, glycerol as carbon source represents the "restrictive" condition. This type of arrest is seen under conditions of nutrient starvation (Hartwell 1974) but it is important to note that this does not normally lead to sporulation, in fact sporulation is inhibited in certain cell cycle mutants which show arrest in G<sub>1</sub> (e.g. cdc28 homozygous diploids, Simchen 1974). Therefore it is clear that the site of the spd1 lesion is likely to represent a different previously unidentified function in the cell cycle control.

Isolation of the cdc28 cell cycle mutant has been important in establishing the order of events during this part of the cell

cycle (Pringle and Maddox 1974 ). The cdc 28 event occurs very early in G<sub>1</sub>; nutritional arrest is expressed prior to or at the step in the cell cycle that is both sensitive to  $\alpha$  mating factor and mediated by the cdc 28 gene product. Cells carrying the cdc 28 marker were subjected to nutrient starvation at the permissive temperature. On a shift to nutrient medium and the restrictive temperature, these cells did not undergo a further budding cycle. As cells are committed to undergo the mitotic cell cycle after the cdc 28 mediated event has been executed ( Hartwell 1974) it follows that the nutrient deprivation must be monitored at or prior to the step mediated by the cdc 28 gene product. Following the completion of the work for this thesis mapping of the spd1 lesion in relation to the cdc 28 mutation was carried out in a similar manner using an spd1 cdc 28 haploid strain. The double mutant was subjected to glycerol medium at the permissive temperature for the cdc 28 gene function then transferred to glucose medium ( permissive for the spd1 function) and the restrictive temperature for the cdc 28 function. No further budding occurred in the double mutant indicating that the spd1 mutation arrested cells at or probably before the execution point of the cdc 28 gene function. The spd1 locus has been mapped; it lies 19cM to the left of the centromere on chromosome XV and is unlinked to cdc 25 or cdc 28 loci (I.W. Dawes Pers. Comm.).

Much of the support for an alteration in the spd1 mutants of some event in the G<sub>1</sub> phase of the cell cycle comes from studies on growth and morphology of spd1 mutants and their revertants under different nutritional conditions. No biochemical alterations have been defined, but it is interesting to note that much of

the available information on the cell cycle is based on observation of morphology of specific mutants.

The influence of different carbon sources on the time spent by cells in different stages of the cell cycle has been examined by Barford and Hall (1976). Estimates obtained indicate that the lengths of the S period and mitosis are relatively independent of carbon source, whilst the lengths of  $G_1$  and  $G_2$  phases show considerable variation. The length of time spent in  $G_1$  increases with different carbon sources in the order glucose < maltose << ethanol. Therefore it is conceivable that if the spd1 mutants are altered in some aspect of the transition from mitosis to meiosis, the longer the time spent in  $G_1$ , the greater the chance that the initiation of the developmental programme might occur. Certainly, the ability of the wild-type to grow on acetate, glycerol and pyruvate is much less than on ethanol medium. Therefore it is probable that the length of  $G_1$  would be considerably extended on these carbon sources. In fact Jagadish and Carter (1977) propose that it is the  $\lambda$  period in  $G_1$  prior to the expression of the cdc 28 mediated step that varies most with growth rate. As the spd1 has been shown to arrest during this time, this must be the period in  $G_1$  involved in the transition from mitosis to meiosis, and therefore there may be a high probability of sporulation being initiated in the spd1 mutants under these growth conditions.

spo 0 revertants may be blocked in the normal events in the cell cycle leading to this transition. The clumpy, abnormally budded appearance of stationary phase revertant cells is not characteristic of stationary phase cells which usually show  $G_1$  arrest (i.e. accumulation as large but normally shaped and unbudded cells), neither

is it characteristic of the spo 0 revertants themselves during normal logarithmic growth. Their appearance in stationary phase is highly suggestive of an inability to undergo a normal arrest in G<sub>1</sub> in response to starvation conditions. As the cdc 28 event appears to affect "start" in the cell cycle, cells being committed to the completion of the cell cycle after this, these revertant species may be altered in the cdc 28 site or in a gene whose function is to regulate "start". In fact the cdc 28 strain at the restrictive temperature shows a similar abnormal phenotype to spo 0 mutants. The rapid loss of viability of the spd1 revertants may be due to a lethal effect of abnormal mitosis. Indeed, Hartwell (1974) has suggested that arrest of starved cells in G<sub>1</sub> may permit maintenance of viability for longer periods than arrest in other periods of the cell cycle.

Some alleles of the spo 0 site do however sporulate at a very low frequency. Those cells which do sporulate do not represent secondary revertants of the original reversion event, since re-germination of these spores leads to strains which are also poor sporulating. These therefore are analagous to the oligosporogenous mutants found in Bacillus subtilis (Schaeffer *et.al.* 1965; Piggot and Coote 1976).

The phenotypes of the spd1 mutants and their spo0 revertants therefore point to a change in the regulation of events in the early G<sub>1</sub> phase of the cell cycle. Very recently, Shilo *et.al.* (1978) have analysed sporulation in homozygous diploids of certain mutations affecting "start" in the cell cycle. Two of these, cdc25 and cdc35, which both map at or before cdc28 showed similar derepressed sporulation in rich media at the restrictive temperature

to the spd1 mutants. The spd1 lesion is unlinked to the cdc25 locus but its relationship to the cdc35 locus has not yet been investigated. Although no true temperature sensitive spd1 alleles have yet been isolated, the phenotypic similarity between the two types of mutant provide strong evidence for an alteration in the same area of control in both cases.

In conclusion therefore, while it remains possible that the expression of the spd1 lesion arises from some disruptive effect on energy metabolism, this must be a unique effect as normally starvation does not lead to sporulation but to an accumulation in  $G_1$ . In a way, the failure to identify any biochemical defect in either the spd1 mutants or the spo0 revertants is positive evidence for a primary alteration in cell cycle control. One can therefore tentatively propose a hypothesis for the mechanism of regulation of sporulation.

Evidence has been presented which indicates that sporulation is sensitive to repression by both carbon and nitrogen sources. However, bearing in mind that the spd1 phenotype is not expressed on all non-fermentable carbon sources, only on those on which the wild-type itself grows slowly, it is possible that initiation of meiosis is governed by the level of an effector molecule which reflects the rate of some part of metabolism (dependant on both carbon and nitrogen source) for example protein synthesis. In fact, Unger and Hartwell (1976) suggest that the signal for all types of starvation leading to  $G_1$  arrest is a common one and lies at the level of protein biosynthesis. It is not unreasonable to propose that such a low molecular weight effector as well as regulating nutritional control of cell division may also control

initiation of sporulation by interacting with the same or a different receptor protein.

Different levels or thresholds of this effector molecule may serve to regulate a receptor protein to direct the cell into one of three possible pathways, mitosis,  $G_1$  arrest or sporulation. Alternatively a certain level of effector molecule could control expression of a protein specifically involved with initiation of sporulation. Two possibilities exist:

- 1) either the metabolism of the spd1 mutants is altered on poor carbon sources in such a way that levels of this effector molecule are capable only of directing cells into the sporulation sequence or
- 2) more probably the receptor protein itself is altered and at slow growth rates has reduced affinity for low levels of an effector molecule. This nutrient availability control if it acts at the transition point between mitosis and meiosis could in the spd1 mutants be altered in such a way that the effect of the nitrogen source was overruled under some circumstances.

Observed repression of sporulation by exogenous glucose or ammonium may arise not only at initiation but also by action on processes which are essential, but not specific, to sporulation. For example glucose may influence the activity of TCA cycle enzymes which are necessary for sporulation: ammonium ion may have an effect on protease activity, and appears to have an **inhibitory** effect on pre-meiotic DNA synthesis.

The spo0 revertants on the other hand, apparently fail either to sporulate or accumulate in  $G_1$  under starvation conditions. They may be altered in one of the events constituting "start" in the cell cycle. A structural change in a protein which responds

to nutrient availability and acts to regulate "start" may prevent normal arrest in  $G_1$ . In such a situation sporulation may still be able to proceed in a low percentage of the population dependent on the severity of the lesion.

Clearly, given appropriate control mutants altered in their sensitivity to catabolite repression of enzyme synthesis (e.g. mutants derepressed for carbon catabolite repressible enzyme synthesis) an ideal situation exists for testing the specificity of a glucose effect on sporulation. Similarly mutants altered in their nitrogen repression profiles would be equally interesting.



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THE REGULATION OF SPORULATION IN *SACCHAROMYCES CEREVISIAE*:  
GENETIC AND BIOCHEMICAL STUDIES ON  
THE ROLE OF NITROGEN REPRESSION

J. H. Kinnaird and I. W. Dawes

*Department of Microbiology,  
University of Edinburgh,  
Edinburgh, Scotland.*

SUMMARY

*Spd* mutants of *Saccharomyces cerevisiae* which do not respond to nitrogen repression of sporulation, may have lost the nitrogen control of initiation. These mutants have been characterized genetically and have been found to be recessive to the wild-type. They also fail to complement one another and are therefore likely to fall into the same complementation group. Analysis has shown that there is a high degree of linkage between the mutations. They may therefore represent alleles of the same gene.

The nitrogen-repression profile of arginase and NAD-specific glutamate dehydrogenase show that this site at least, does not represent a common control locus in nitrogen repression of enzyme synthesis and nitrogen repression of sporulation. It seems, therefore that this mutation represents a locus which is specific and essential to the nitrogen regulation of initiation of sporulation.

INTRODUCTION

Meiosis and sporulation of yeast are convenient systems for use in the study of the regulation of cell development. Examination of genetic control is facilitated by the existence of meiosis and, in addition, the sporulation sequence is well characterized. The conditions leading to the relief of "catabolite repression" of sporulation are similar to those established in bacterial systems (Schaeffer *et al.* 1965; Miller, 1963; Dawes and Mandelstam, 1970), however, both carbon and nitrogen sources separately, repress sporulation in *Saccharomyces cerevisiae* whereas in *Bacillus* relief of either

carbon or nitrogen repression leads to sporulation (Dawes, 1975).

We are interested in this initiation process and the isolation of mutants possibly altered in the nitrogen repression of sporulation has recently been reported (Dawes, 1975). The mutants of *Saccharomyces cerevisiae*, designated *spd* (spore derepressed), show poor growth but extensive sporulation on carbon sources other than glucose, in particular, non-fermentable ones such as glycerol and acetate. They also show high sporulation after exhaustion on a rich medium containing glucose, on which the wild-type shows no sporulation. From data obtained using minimal media, it appeared that the mutants were insensitive to nitrogen source concentration as regards initiation of sporulation, but glucose control was still effective, i.e. nitrogen source repression of sporulation has been lost. As such, these are possibly regulatory mutants.

In yeast, there are enzymes which are subject to nitrogen repression, and it may be that there exists a common regulatory element in the nitrogen repression of both sporulation and inducible enzyme synthesis. We have therefore examined these mutants for their response to nitrogen source repression of inducible enzyme synthesis to see if there is any link between the two systems. In addition, the mutants have been characterized genetically to determine whether they exist at one locus or at several, and, to find their dominance relationships.

## MATERIALS AND METHODS

### *Strains*

Strains of *Saccharomyces cerevisiae*, their genotypes and characteristics are listed in Table I.

16D and 19D were isolated after UV mutagenesis followed by an ether selection technique, which effectively selects against vegetative diploids, and re-cycling procedures (Dawes and Hardie, 1974). The use of homothallic strains permits the isolation of homozygous recessive mutations which otherwise would not be recovered (Esposito and Esposito, 1969). 7.4A was isolated by  $\gamma$ -irradiation of wild-type DB1, using the same selection procedure.

### *Growth Conditions*

Strains were maintained on YEPD agar, containing 2% glu-

TABLE I

*Strains of Saccharomyces cerevisiae*

Strain	Source	Genotype	Characteristics
S41	Dr. H.O. Halvorson	<u><i>a D arg4-1 cyh1</i></u> <u><i>α D arg4-1 cyh1</i></u>	Wild-type, sporulation subject to carbon and nitrogen repression
DB1	Derivative of S41	<u><i>a D his4</i></u> <u><i>α D his4</i></u>	Wild-type, sporulation subject to carbon and nitrogen repression
16D	Derivative of S41	<u><i>a D arg4-1 cyh1 spd1-1</i></u> <u><i>α D arg4-1 cyh1 spd1-1</i></u>	Derepressed for sporulation, subject to carbon repression only, does not grow on glycerol
19D	Derivative of S41	<u><i>a D arg4-1 cyh1 spd1-2</i></u> <u><i>α D arg4-1 cyh1 spd1-2</i></u>	Derepressed for sporulation, subject to carbon repression only, does not grow on glycerol
7.4A	Derivative of S41	<u><i>a D his4 spd1-3</i></u> <u><i>α D his4 spd1-3</i></u>	Derepressed for sporulation, subject to carbon repression only, poor growth on glycerol

Sporulation in *S. cerevisiae*

cose, 2% Bacto-Peptone (Difco), 1% yeast extract (Difco) and 2% agar. Cultures were pre-grown with shaking at 30° on liquid YEPD to early stationary phase (about 24 hours) and inoculated to a turbidity of 0.1 at 600 nm, into various synthetic minimal media containing Wickerham minimal salts (Difco) and 2% glucose. Auxotrophic requirements were added at a concentration of 20  $\mu\text{g ml}^{-1}$ . Nitrogen sources were used as tabulated in the results section (Table IV and V).  $(\text{NH}_4)_2\text{SO}_4$  was used at 0.1%, arginine-HCL and sodium glutamate at 20 mM concentrations. Strains were sporulated for meiotic analysis on plates containing potassium acetate at 2%, auxotrophic requirements at 20  $\mu\text{g ml}^{-1}$  and 2% agar.

### *Genetic Analysis*

This was carried out using the standard genetic techniques of Mortimer and Hawthorne (1969). Crosses between homothallic diploids were done by mixing on YEPD plates, cultures previously sporulated on potassium acetate, and selecting for diploids on minimal medium.

### *Preparation of Samples and Enzyme Assay Procedures*

Cultures for assay were harvested at a turbidity of 1.0 by filtration of suitable aliquots on Millipore membrane filters and washed three times with 10 ml volumes of ice-cold distilled water. The filters were frozen and the cells made permeable by freeze-drying. Enzyme assays were performed on these whole cells, following resuspension in the assay buffer. Arginase (L-arginine ureohydrolase EC 3.5.3.1) was assayed by the method of Middelhoven (1964) using heat and manganese activation. Urea formed was estimated by the colorimetric diacetyl-monoxime method of Moore and Kauffman (1970). Specific activity is expressed as  $\mu\text{mol urea produced h}^{-1} (\text{mg protein})^{-1}$ . NAD<sup>+</sup>-specific glutamate dehydrogenase (L-glutamate:NAD oxidoreductase {deaminating} EC 1.4.1.2) was measured spectrophotometrically by following the oxidation of NADH at 340 nm (Ferguson and Sims, 1972). Specific activities are given in terms of unit absorbance change  $\text{h}^{-1} (\text{mg protein})^{-1}$ . Protein was estimated by the method of Lowry *et al.* (1951). Bovine serum albumin was used as standard.

## RESULTS

### *Genetic Characterization*

The results of a dominance and complementation study be-

tween the mutants and wild-type are presented in Table II. The data shows that the three mutations are recessive to wild-type and appear to belong to the same complementation group, although 7.4A can be seen to have complemented 19D to some extent.

TABLE II

*Dominance and Complementation Data*

	% Sporulation					
	S41	DB1	16D	16D9A	19D	7.4A
S41	0					
DB1	0	0				
16D	-	0	30			
16D9A	0	-	-	35		
19D	-	0	-	30	25	
7.4A	0	0	25	-	1	50

Sporulation characteristics of diploid strains from crosses indicated. The figures shown represent the percentage sporulation after 72 h incubation with shaking at 30° in liquid YEPD. Percentage sporulation was estimated by direct counting of asci; 0 = no sporulation; - = crosses which were not carried out. 16D9A is a homozygous diploid, requiring histidine, and was derived from a cross of 16D to a haploid *his4* tester strain.

Linkage of the three mutations to each other was established from the segregation patterns obtained from dissection of the crosses indicated in Table III. All asci obtained were parental ditypes, indicating that there is a high degree of linkage between these three mutations. They are therefore considered to represent alleles at the same locus affecting nitrogen repression of sporulation and are designated *spd1-1*, *spd1-2* and *spd1-3* for mutations in strains 16D, 19D and 7.4A respectively.

*Nitrogen Repression of Enzyme Synthesis*

Biochemical analysis of nitrogen repression of enzyme synthesis was carried out by examining induction and repression profiles for arginase and NAD<sup>+</sup>-specific glutamate dehydrogenase. Both enzymes are strongly subject to repression

TABLE III  
*Linkage Analysis Data*

Cross	PD	NPD	T
16D x 7.4A	6	0	0
19D x 7.4A	6	0	0

PD = parental ditype; NPD = non-parental ditype; T = tetra-type. Six complete asci were obtained from each dissection.

TABLE IV  
*Induction and Repression of Arginase in Wild-Type and Sporulation-Derepressed Strains*

Growth Conditions	S41	Specific Activity*		
		7.4A	16D	19D
Glucose + $\text{NH}_4^+$	11.0	9.5	9.0	6.0
Glucose + $\text{NH}_4^+$ + arginine	39.0	30.5	22.0	23.5
Glucose + arginine	57.5	59.5	53.5	43.0

\*Specific activity is represented as  $\mu\text{mol}$  urea produced  $\text{h}^{-1}$  ( $\text{mg protein})^{-1}$ .

TABLE V  
*Induction and Repression of NAD-Specific Glutamate Dehydrogenase in Wild-Type and Sporulation-Derepressed Strains*

Growth Conditions	S41	% Sporulation*		
		7.4A	16D	19D
Glucose + $\text{NH}_4^+$	1.2	1.8	2.1	1.5
Glucose + $\text{NH}_4^+$ + Glutamate	2.0	2.4	2.6	2.1
Glucose + Glutamate	9.0	11.4	10.2	7.2

\*Specific activity is represented as unit absorbance change  $\text{h}^{-1}$  ( $\text{mg protein})^{-1}$ .

by ammonium ions. The levels of these enzymes present in wild-type and mutant strains under different growth conditions are given in Tables IV and V. These include basal levels (growth on glucose and ammonium), induced levels (growth on glucose and arginine or glutamate) and ammonium repressed levels in the presence of inducer.

In the wild-type strain S41, arginase was inducible 5 to 6 fold by growth on arginine as sole nitrogen source. Ammonium at 0.1% repressed synthesis to approximately half of the induced level. Growth on glutamate led to a 7-fold induction of NAD<sup>+</sup>-specific glutamate dehydrogenase, but for this enzyme the ammonium repression effect was greater than for arginase, i.e. it appears to be subject to stronger repression by ammonium.

It can also be seen that both enzymes in all three mutants were subject to the same NH<sub>4</sub><sup>+</sup>-mediated repression of their synthesis as in the wild-type strain. This is in strong contrast to the effect of ammonium on sporulation reported previously for two of these mutant strains (16D and 19D) and for strain 7.4A (unpublished observations).

#### DISCUSSION

The mutants described here are altered in some way, in nitrogen repression, since they sporulate regardless of the nitrogen concentration in the medium, whereas, wild-type strains sporulate under conditions in which the carbon source is non-repressing and nitrogen source is absent (Croes, 1967).

The genetic analysis indicated that the mutants are recessive to wild-type and that they all fall into the same complementation group. Meiotic analysis also showed that they are very closely linked. All three mutants probably represent alleles of the same gene and, as such, are designated *spd1-1*, *spd1-2* and *spd1-3*. The fact that 7.4A appears to complement 19D to some extent is perhaps due to intragenic complementation which would mean that the locus may be complex.

It seems feasible that there may be a factor in the control pattern which is common to both repression of sporulation and these inducible enzymes sensitive to nitrogen repression of their synthesis, particularly as both appear to be similar phenomena. However, under the specific experimental conditions used here, there seems to be no effect of the *spd* mutation on regulation of nitrogen repressible enzyme synthesis.

There are several possibilities which could give rise

to this result. One is that the mutants are defective in a common nitrogen repression mechanism not affecting the two enzymes studied here (arginase and NAD-specific glutamate dehydrogenase). Illustrating this possibility is the work of Dubois *et al.* (1973) on mutants lacking NADP-specific glutamate dehydrogenase. This lesion causes certain enzymes, but not all, to lose their sensitivity to ammonium repression.

The second possibility is that the *spd* mutation represents a function which is specific to sporulation and essential for its control. This appears more likely, since we have found no difference in the repression characteristics of two widely different enzymes, in three mutants. It is also possible that if the *spd1* locus itself, is specific to sporulation, it may still be part of a general repression mechanism which also acts on enzyme synthesis. However, it is known that mutation to the nitrogen derepressed phenotype is an extremely rare event (occurring in less than 1 in  $10^8$  survivors after UV mutagenesis), and so far, only one site has been found which produces this effect. This latter theory could therefore be less likely.

It is known that replacing growth medium to sporulating cells can reverse sporulation up to a certain point of commitment, after which sporulating cells do not revert to growth, but complete sporulation (Ganeson *et al.* 1958). Yeast sporulation is inhibited by ammonium compounds during the sporulation sequence (Miller, 1971). The existence of derepressed mutants which can initiate and complete sporulation in the presence of high ammonium concentrations normally repressing sporulation, indicates that a single mutation radically alters the nitrogen repression profile, and that there is only one main mechanism of ammonium repression affecting all processes essential to sporulation.

In summary, the *spd1* mutants have lost nitrogen control of sporulation probably by virtue of a lesion in a control gene specific to sporulation. Derepressed phenotypes are very useful tools, since in such mutants, the lesion(s) are restricted to control functions responsible for initiation of the developmental sequence, which is itself unaltered. An analogous situation which illustrates this is seen in the lytic development of bacteriophage lambda. Derepressed mutants exist which are constitutively lytic. These are defective in very few control genes; those associated with production of phage repressor protein (CI, CII, CIII) or, more rarely double mutants at operator sites ( $O_L$  and  $O_R$ ) at which the repressor acts (Ptashne, 1971).



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